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(71) Applicant:
F. HOFFMANN-LA ROCHE AG
4070 Basel (CH)

(72) Inventors:
- **Brugger, Roland**
  79539 Lörrach (DE)
- **Lehmann, Martin, Dr.**
  Princeton, NJ 08540 (US)
- **Wyss, Markus**
  4410 Liestal (CH)

(74) Representative: **Braun, Axel et al**
F.Hoffmann-La Roche AG
Patent Department (PLP),
124 Grenzacherstrasse
4070 Basel (CH)

(54) Phytase formulation

(57) A stabilized enzyme formulation is disclosed which comprises phytase and at least one stabilizing agent selected from the group consisting of:

a) C_5 sugars such as xylitol and ribitol,

b) polyethylene glycol having a molecular weight of 600 to 4000 Da,

c) the disodium salts of malonic, succinic and glutaric acid, and

d) carboxymethylcellulose, and

e) sodium alginate.

Alternatively, phytase may be stabilized by chemical crosslinking with either

a) glutaraldehyde, or

b) oxidation of phytase carbohydrate residues with sodium periodate and subsequent addition of adipic acid dihydrazide.

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Description

[0001] The present invention relates to liquid and dry phytase formulations having an increased stability, preferably thermostability, which is obtained by the addition of stabilizing agents, or by crosslinking.

[0002] Although a large amount of phosphate is present in feed in form of phytate phosphorus, monogastric animals, like pigs and poultry, lack the ability to use this form of phosphate. The alkali or earth alkali salts of phytic acid occur naturally mainly in cereals. Since monogastric animals are not able to use this form of phosphate it is common practice to add phosphate to animal feed.

[0003] On the other hand an enzyme called phytase (*myo*-inositol hexakisphosphate phosphohydrolase) is known to occur in plants and in some microorganisms. Since phytase can be produced by fermentation it is known in the art to use phytase as an animal feed additive in order to enhance the nutritive value of plant material by liberation of inorganic phosphate from phytic acid (*myo*-inositol hexakisphosphate). By adding phytase to the animal feed the level of phosphorus pollution of the environment can be reduced since the animal is able to make use of the phosphate liberated from phytate by the use of phytase.

[0004] For feed application a stable preferably thermostable phytase is of general interest in order to avoid problems that may occur during the formulation (e.g. spray drying, granulation) and feed treatment processes (e.g. pelleting, extrusion, expansion) where temporarily high temperatures (up to 80-120 °C) and shear stress may affect the protein structure and lead to an undesired loss of activity.

[0005] The international patent application WO 93/16175 of Gist-Brocades describes stabilized liquid formulations of phytase. It is suggested to use as stabilizing agent urea and a water-soluble polyol whereby sorbitol, glycerol and polyethylene glycol having a molecular weight of 6000 are mentioned.

[0006] It is an object of the present invention to improve the stability, preferably rhermostability of phytase whereby stability is defined as the ability to retain activity under various conditions. This stability aspect relates to the entire life cycle of the enzyme which comprises production (fermentation, downstream processing, formulation and heat treatment of feed), distribution (transport and storage) and final application. For a commercially interesting enzyme like phytase it is important to withstand the high temperatures reached during various feed treatment processes like pelleting, extrusion and expansion (up to 80-120 °C) and to be stable during long-term storage.

[0007] The term "stability" as used in the present invention relates to all the specifications of an industrial enzyme which comprise aspects such as activity, specificity, shelf stability, mechanical stability, microbial stability, toxicity, chemical composition and physical parameters such as density, viscosity, hygroscopy, but also colour, odour and dust. A preferred aspect of the present invention relates to the stability of phytase against thermal inactivation during formulation and feed treatment processes such as pelleting, extrusion and expansion.

[0008] A major barrier to the wide use of phytases is the constraint of thermal stability (80-120 °C) required for these enzymes to withstand inactivation during feed treatment processes. The currently available industrial phrases all originate from *A. niger* and have a low intrinsic resistance to heat inactivation. As an alternative or in addition to molecular biological approaches the present invention enhances the stability, preferably thermostability of a protein by the addition of different additives and in another aspect by the chemical crosslinking of enzyme monomers to oligomers.

[0009] The experiments leading to the present invention were also performed with the so-called consensus phytase, a phrase developed according to a theoretical molecular biological approach which has a higher intrinsic stability compared with *Aspergillus* phytases, see European Patent Application Publication No. 897 985. In the practice of the present invention the consensus phytases specifically described in examples 3 - 13 can also be used.

[0010] The present invention discloses the use of different additives which act as stabilizing agent on the stability, preferably thermostability of the enzyme.

[0011] Regarding the temperature dependence of the specific activity of the non-formulated phytases which can preferably be used in the present invention three different groups can be formed according to their activity maximum. The activity maximum is reached at the following temperatures: for *A. fumigatus and A. niger* phytase at 55 °C, for *A. terreus* CBS and *A. nidulans* phytase at 45 °C and for consensus phytase at 65 °C. A temperature of 10-15 °C above the determined temperature maximum - where the non-formulated phytases were completely inactive - was chosen as screening point for studying the effect of the stabilizing agents on the thermostability of phytases, i.e. 60 °C for *A. nidulans* and *A. terreus* CBS phytase, 65 °C for *A. niger* and *A. fumigatus* phytase, and 75 °C for consensus phytase.

[0012] The present invention provides a stabilized, preferably thermostabilized enzyme formulation comprising phytase and at least one stabilizing agent selected from the group consisting of:

a) polyols containing five carbon atoms, preferably C_5 sugars, more preferably xylitol or ribitol,

b) polyethylene glycol having a molecular weight of 600 to 4000 Da,

c) the disodium salts of malonic, glutaric and succinic acid,

d) carboxymethylcellulose, and

e) sodium alginate

[0013] The present invention also provides a stabilized, preferably thermostabilized enzyme formulation comprising phytases which have been crosslinked:

a) by chemical reactions with glutaraldehyde; or by
b) oxidation with sodium periodate and subsequent addition of adipic acid dihydrazide

[0014] Although it would be possible to use other phytases obtained from other sources than microorganisms it is preferred to use a phytase which has been produced by microorganisms. In the present invention preferably such phytases are used which are produced by a fungus, and more preferably from the group consisting of *Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus terreus*, and *Aspergillus niger*. Another phytase preferably used in this invention is the so called consensus phytase. It is, however, also possible to produce such phytases by genetic engineering whereby the gene obtained from a fungus is transferred to a host organism like a bacterium (e.g. *E.coli*), a yeast or another fungus, for further details, see e.g. European Patent Application Publication No. 684313 and European Patent Application Publication No. 897 010.

[0015] The term enzyme formulation comprises all liquid and dry formulations in which the enzyme phytase may be commerciallized. Preferably, the source of phrase for such a formulation is a rather raw, liquid preparation obtained from the fermentation broth. For the preparation of a liquid phytase formulation the selected stabilizing agents are added or the phytase is crosslinked. To obtain a stabilized, preferably thermostabilized dry formulation the phrase is a) spray dried or granulated in the presence of the selected stabilizing agents, or b) chemically crosslinking.

[0016] In one preferred embodiment the liquid enzyme formulation comprises as stabilizing agent polyethylene glycol whereby the polyethylene glycol is present in a concentration of 10-50% (w/w) in the final formulation.

[0017] Preferably the enzyme formulation comprises polyethylene glycol having a molecular weight of 1000-3350 Da. It is especially preferred to use a polyethylene glycol having a molecular weight of about 1450. Polyethylene glycols with molecular weights slightly outside of the preferred range (600 Da and 4000 Da, respectively) showed still reasonable effect but are less preferred. The stabilizing effect of polyethylene glycol was shown to be molecular weight-dependent (see Figures 2 and 3).

[0018] In another preferred embodiment of the present invention the stabilizing agent is xylitol or ribitol. Both are sugar alcohols having a five carbon atom structure. Xylitol and ribitol are preferably used in a concentration of 20 to 60% (w/w) in the final liquid formulation. Surprisingly xylitol and ribitol as stabilizing agents of, e. g. , *A. fumigatus* phytase increased the specific activity measured at 65 °C to 11-12 U/mg at a concentration of 12.5%, and to 51-90 U/mg at a concentration of 25% of the polyol (see Figure 4).

[0019] In another embodiment of the present invention the liquid enzyme formulation comprises as stabilizing agent the disodium salts of glutaric, succinic or malonic acid whereby the concentration of the salt in the final formulation ranges between 10 and 30% (w/w). The addition of malonate, succinate and glutarate at a concentration of 25% resulted in a significant increase in *A. fumigatus* phytase thermostability with considerable activity still being detected at 70 °C for malonate and 65 °C for succinate and glutarate as can be seen in Figure 6.

[0020] In addition thereto the carboxylates stimulated *A. fumigatus* phytase activity measured at 37 °C with an approximately 4-fold increase in phytase activity beeing observed in the case of malonate, a 2-fold increase for succinate and minor effects for glutarate. Investigation of different concentrations (5, 10 and 25%) of malonate showed that thermostabilization of *A. fumigatus* phytase is concentration-dependent whereas stimulation of enzymatic activity, at least in this concentration range, is not (see Figure 7). In contrast to these findings different concentrations (5, 10 and 25%) of sodium acetate which is a monocarboxylic acid, caused an up to 2-fold increase in specific activity of *A. fumigatus* phytase at 37 °C, but had only minor effects on the thermostability of the protein (see Figure 8). Therefore, it may be concluded that carboxylate groups are responsible for activity modulation whereas bifunctional dicarboxylates stabilize phytases possibly by ionic interactions. The sodium malonate and succinate generally increased the thermostability of *A. nidulans*, *A. terreus* CBS, *A. niger* and consensus phytase by 5-15 °C. On the other hand stimulation of phytase activity was only observed for *A. nidulans* and *A. fumigatus* phytase both having rather low specific activity but not for *A. terreus* CBS, *A. niger* and consensus phytase (see Figures 9 and 10).

[0021] In another embodiment of the present invention the enzyme formulation comprises as stabilizing agent the polymers carboxymethylcellulose and/or sodium alginate whereby the concentration in the final liquid formulation is between 1 and 20% preferably 1 and 10% (w/w). The addition of these polymeres to *A. fumigatus* phytase preparations resulted in a significant 5 to 10% increase of phytase thermostability.

[0022] In another embodiment of the present invention the enzyme formulation comprises as stabilizing agent alginate, preferably sodium alginate and most preferably in a concentration of 1 to 10% (w/w) in the final liquid formulation.

[0023] In a further embodiment of the present invention the enzyme formulation comprises crosslinked phytase. For the preparation of such a stabilized phytase form, glutaraldehyde is added to the phytase at a concentration resulting in an oligomerization of the protein.

[0024] In another embodiment the enzyme formulation comprises phytase which has been crosslinked via its carbohydrate chains. Crosslinking involves as a first step periodate oxidation of the carbohydrate residues followed by reaction of the generated aldehyde groups with adipic acid dihydrazide.

[0025] Depending on the conditions employed, the crosslinking reaction can lead to various derivatives of an enzyme, namely

a) modified enzyme molecules that have reacted with only one hydrazide group of adipic acid dihydrazide,

b) intramolecularly crosslinked enzymes, with or without intermolecular crosslinking, and

c) intermolecularly crosslinked, soluble oligomers or insoluble polymers.

[0026] In most cases the reaction results in a mixture of several forms. Crosslinking of *A. fumigatus* and consensus phytase both expressed in *Hansenula polymorpha* resulted in the formation of oligomeric forms. The degree of crosslinking could be controlled effectively by changing the degree of enzyme oxidation. An optimal thermostabilization of phytase has been observed at a concentration of 50 mM sodium periodate applied to a 5 mg/ml phytase solution. For both phytases an increase in thermostability between 10 and 15 °C has been observed (see Figure 12). It should be noted that the oxidized phytases formed a significant amount of dimers, trimers and tetramers even without addition of adipic acid dihydrazide (see Figure 11A).

[0027] Another aspect of the present invention concerns the use of the listed stabilizers as additives for the production of dry/solid phytase formulations. In this embodiment of the present invention the addition of stabilizers (1 to 20% (w/w) of xylitol/ribitol, 1 to 20% (w/w) of polyethylene glycols with a molecular weight preferably between 1000 and 3350 Da and/or 1 to 20% (w/w) of dicarboxylates like malonate, succinate and glutarate, and/or 1 to 10% (w/w) of the polymers carboxymethlycellulose and/or alignate, preferably sodium alginate disolved in 100-200 ml phytase liquid (crosslinked or non-crosslinked) or added as solid compounds to the standard granulation mixture (containing ligninsulfonat as binder, silica and gipsum as carrier) Such formulation can result in an increased recovery (up to 20%) of phytase activity determined after a high shear granulation process which included a drying step of the granulates on a fluid bed dryer at 45°C for 15 mm. In addition such granulates which contain stabilizers can show, when mixed with feed, an increased recovery of enzymatic activity after the feed treatment (e.g. a pelleting process at 85°C) compared to granulates without such additives.

[0028] Another aspect of the present invention concerns methods of preparing feed compositions for monogastric animals, whereby the feed is supplemented with a thermostabilized dry or liquid enzyme formulation according to any of claims (1-13). The phytase supplemented feed can be subjected on several methods of feed processing like extrusion, expansion and pelleting, where temporarily high temperatures may occure and thermostabilization is an advantage.

[0029] The stabilized enzyme formulation of the present invention can be appllied for example on feed pellets. The thermostabilized liquid enzyme formulation may be diluted with tap water to yield a solution having the desired activity of phytase (100 - 200 phytase units/g solution). The feed pellets can be transferred to a mechanical mixer and the diluted enzyme formulation is sprayed onto the feed pellets while being agitated in order to yield a homogeneous product with an added phytase activity of for example 500 units phytase/kg feed pellets. Alternatively the dry or liquid enzyme formulation can be directly mixed with the mash feed before this mixture is then subjected to a process such as pelleting, expansion or extrusion.

[0030] In a further aspect the present invention concerns a method of providing a monogastric animal with its dietary requirement of phosphorus wherein the animal is fed with a feed according to the present invention and whereby no additional phosphorus is added to the feed.

[0031] The results of the experiments of the present invention are shown in the following Figures.

Figure 1. Comparison of the temperature dependence of activity of *A. fumigatus, A. nidulans, A. terreus* CBS, *A. niger* and consensus phytase measured under standard assay conditions as described in Example 1.

Figure 2. Effect of different polyethylene glycols on the specific activity of *A. fumigatus* phytase at 65 °C.

Figure 3. Effect of 50% solutions of polyethylene glycols with different molecular weights on the thermostability of *A. niger*, consensus, *A. terreus* CBS and *A. nidulans* phytase. The specific activities were measured at 60 °C for *A. terreus* CBS and *A. nidulans* phytase, at 65 °C for *A. niger* phytase and at 75 °C for consensus phytase.

Figure 4. Effect of 25 and 50% solutions of different polyols on the specific activity of *A. fumigatus* phytase at 65 °C.

Figure 5. Temperature dependence of activity of *A. niger* (A), consensus (B), *A. nidulans* (C) and *A. terreus* CBS (D) phytase in the presence of 50% xylitol as additive.

Figure 6. Temperature dependence of activity of *A. fumigatus* phytase in the presence of 25% concentrations of disodium malonate, succinate and glutarate.

Figure 7. Temperature dependence of activity of *A. fumigatus* phytase in the presence of 5, 10 and 25% disodium malonate.

Figure 8. Temperature dependence of activity of *A. fumigatus* phytase in the presence of 5, 10 and 25% sodium acetate.

Figure 9. Temperature dependence of activity of *A. niger* (A), consensus (B), *A. terreus* CBS (C) and *A. nidulans* (D) phytase in the presence of 25% disodium malonate.

Figure 10. Temperature dependence of activity of *A. niger* (A), consensus (B), *A. terreus* CBS (C) and *A. nidulans* (D) phytase in the presence of 25% disodium succinate.

Figure 11.

A) SDS-PAGE of *A. fumigatus* phytase samples after incubation with different concentrations of sodium periodate.

B) SDS-PAGE of the different oxidized *A. fumigatus* phytase samples from (A) after subsequent crosslinking with adipic acid dihydrazide.

Figure 12 Temperature dependence of activity of *A. fumigatus* phytase and consensus phytase before and after crosslinking with periodate/adipic acid dihydrazide.

Figure 13 Design of the consensus phytase sequence. The letters represent the amino acid residues in the one-letter code. The following sequences were used for the alignment: *phyA* from *Aspergillus terreus* 9A-1 (Mitchell et al, 1997; from amino acid (aa) 27), *phyA* from *A. terreus* cbs116.46; (van Loon et al., 1998; from aa 27), *phyA* from *Aspergillus niger* var. *awamori* (Piddington et al, 1993; from aa 27), *phyA* from *A. niger* T213; from aa 27), *phyA* from *A. niger* stain NRRL3135 (van Hartingsveldt et al, 1993; from aa 27), *phyA* from *Aspergillus fumigatus* ATCC 13073 (Pasamontes et al, 1993; from aa 25), *phyA* from *A. fumigatus* ATCC 32722 (van Loon et al, 1998; from aa 27), *phyA* from *A. fumigatus* ATCC 58128 (van Loon *et al.,* 1998; from aa 27), *phyA* from *A. fumigatus* ATCC 26906 (van Loon et al, 1998; from aa 27), *phyA* from *A. fumigatus* ATCC 32239 (van Loon et al, 1998; from aa 30), *phyA* from *Emericella nidulans* (Pasamontes et al, 1997a; from aa 25), *phyA* from *Talaromyces rhermophilus* (Pasamontes et al, 1997a; from aa 24), and *phyA* from *Myceliophthora thermophila (*Mitchell et al, 1997; from aa 19). The alignment was calculated using the program PILEUP. The location of the gaps was refined by hand. Capitalized amino acid residues in the alignment at a given position belong to the amino acid coalition that establish the consensus residue. In bold, beneath the calculated consensus sequence, the amino acid sequence of the finally constructed consensus phytase (Fcp) is shown. The gaps in the calculated consensus sequence were filled by hand according to principals stated in Example 3.

Figure 14 DNA sequence of the consensus phytase-1 gene (*fcp*) and of the primers used for the gene construction. The calculated amino acid sequence (Figure 13) was convened into a DNA sequence using the program BACKTRANSLATE (Devereux *et al.,* 1984) and the codon frequency table of highly expressed yeast genes (GCG program package, 9.0). The signal peptide of the phytase from *A. terreus* cbs.116.46 was fused to the *N*-terminus. The bold bases represent the sequences of the oligonucleotides used to generate the gene. The names of the respective oligonucleotides are alternately noted above or below the sequence. The underlined bases represent the start and stop codon of the gene. The bases written in italics show the two introduced *Eco* RI sites.

Figure 15 Alignment and consensus sequence of five *Basidiomycetes* phytases. The letters represent the amino acid residues in the one-letter code. The amino acid sequences of the phytases from *Paxillus involutus*, phyA1 (aa 21) and phyA2 (aa 21, WO 98/28409), *Trametes pubescens* (aa 24, WO 98/28409), *Agrocybe pediades* (aa 19,

WO 98/28409), and *Peniophora lycii* (aa 21, WO 98/28409) starting with the amino acid residues mentioned in parentheses, were used for the alignment and the calculation of the corresponding consensus sequence called "Basidio" (Example 4). The alignment was performed by the program PILEPUP. The location of the gaps was refined by hand. The consensus sequence was calculated by the program PRETTY. While a vote weight of 0.5 was assigned to the two *P. involutus* phytases, all other genes were used with a vote weight of 1.0 for the consensus sequence calculation. At positions, where the program was not able to determine a consensus residues, the Basidio sequence contains a dash. Capitalized amino acid residues in the alignment at a given position belong to the amino acid coalition that establish the consensus residue.

Figure 16 Design of consensus phytase-10 amino acid sequence. Adding the phytase sequence of *Thermomyces lanuginosa* (Berka *et al.*, 1998) and the consensus sequence of the phytases from five *Basidiomycetes* to the alignment of Figure 13, an improved consensus sequence was calculated by the program PRETTY. Additionally, the amino acid sequence of *A. niger* T213 was omitted, therefore, using a vote weight of 0.5 for the remaining *A. niger* phytase sequences. For further information see Example 14.

Figure 17 DNA and amino acid sequence of consensus phytase-10. The amino acid sequence is written above the corresponding DNA sequence using the one-letter code. The sequence of the oligonucleotides which were used to assemble the gene are in bold letters. The label of oligonucleotides and the amino acids, which were changed compared to those for consensus phytase -1, are underlined and their corresponding triplets are highlighted in small cases. The *fcp*10 gene was assembled from the following oligonucleotides: CP-1, CP-2, CP-3.10, CP-4.10, CP-5.10, CP-6, CP-7.10, CP-8.10, CP-9.10, CP-10.10, CP-11.10, CP-12.10, CP-13.10, CP-14.10, CP-15.10, CP-16.10, CP-17.10, CP18.10, CR-19.10, CP-20.10, CP-21.10, CP-22.10. The newly synthesized oligonucleotides are additionally marked by number 10. The phytase contains the following 32 exchanges: Y54F, **E58A**, D69K, D70G, A94K, N134Q, I158V, S187A, Q188N, **D197N**, S204A, T214L, D220E, L234V, A238P, D246H, T251N, Y259N, **E267D**, E277Q, A283D, **R291I**, A320V, **R329H**, **S364T**, I366V, **A379K**, S396A, **G404A**, Q415E, A437G, A463E. The mutations accentuated in bold letters revealed a stabilizing effect on consensus phytase-1 as tested as single mutation in consensus phytase-1.

Figure 18 Alignment for the design of consensus phytase-11. In contrast to the design of consensus phytase-10, for the design of the amino acid sequence of consensus phytase-11, all *Basidiomycetes* phytases were used as independent sequences using an assigned vote weight of 0.2 for each *Basidiomycetes* sequence. Additionally, the amino acid sequence of *A. niger* T213 was used in that alignment, again.

Figure 19 DNA and amino acid sequence of consensus phytase-1-thermo[8]-Q50T-K91A. The amino acid sequence is written above the corresponding DNA sequence using the one-letter code. The replaced amino acid residues are underlined. The stop codon of the gene is marked by a star (*).

Figure 20 DNA and amino acid sequence of consensus phytase-10-thermo[3]-Q50T-K91A. The amino acid sequence is written above the corresponding DNA sequence using the one-letter code. The replaced amino acid residues are underlined. The stop codon of the gene is marked by a star (*).

Figure 21 DNA and amino acid sequence of *A. fumigatus* ATCC 13073 phytase a-mutant. The amino acid sequence is written above the corresponding DNA sequence using the one-letter code. The replaced amino acid residues are underlined. The stop codon of the gene is marked by a star (*).

Figure 22 DNA and amino acid sequence of consensus phytase-7. The amino acids are written above the corresponding DNA sequence using the one-letter code. The sequence of the oligonucleotides used to assemble the gene are in bold letters. Oligonucleotides and amino acids that were exchanged are underlined and their corresponding triplets are highlighted in small cases. The *fcp*7 gene was assembled from the following oligonucleotides: CP-1, CP-2, CP-3, CP-4.7, CP-5.7, CP-6, CP-7, CP-8.7, CP-9, CP-10.7, CP-11.7, CP-12.7, CP-13.7, CP-14.7, CP-15.7, CP-16, CP-17.7, CP-18.7, CP-19.7, CP-20, CP-21, CP-22. The newly synthesized oligonucleotides are additionally marked by number 7. The phytase contains the following 24 exchanges in comparison to the original consensus phytase: S89D, S92G, A94K, D164S, P201S, G203A, G205S, H212P, G224A, D226T, E255T, D256E, V258T, P265S, Q292H, G300K, Y305H, A314T, S364G, M365I, A397S, S398A, G404A, and A405S.

Figure 23 Differential scanning calorimetry (DSC) of consensus phytase-1 and consensus phytase-10. The protein samples were concentrated to ca. 50-60 mg/ml and extensively dialyzed against 10 mM sodium acetate, pH 5.0. A constant heating rate of 10 °C/min was applied up to 95 °C. DSC of consensus phytase-10 (upper graph) yielded

a melting temperature of 85.4 °C, which is 7.3 °C higher than the melting point of consensus phytase-1 (78.1 °C, lower graph).

Figure 24 Differential scanning calorimetry (DSC) of consensus phytase-10-thermo-Q50T and consensus phytase-10-thermo-Q50T-K91A. The protein samples were concentrated to ca. 50-60 mg/ml and extensively dialyzed against 10 mM sodium acetate, pH 5.0. A constant heating rate of 10 °C/min was applied up to 95 °C. DSC of consensus phytase-10-thermo-Q50T (upper graph) yielded a melting temperature of 88.6 °C, while the melting point of consensus phytase-10-thermo-Q50T-K91A was found at 89.3 °C.

Figure 25 Comparison of the temperature optimum between consensus phytase-1, consensus phytase-10 and consensus phytase-10-thermo-Q50T. For the determination of the temperature optimum, the phytase standard assay was performed at a series of temperatures between 37 and 86 °C. The diluted supernatant of transformed *S. cerevisiae* strains was used for the determination. The other components of the supernatant showed no influence on the determination of the temperature optimum: ∧, consensus phytase-1; ✧, consensus phytase-10; ■, consensus phytase 10-thermo-Q50T.

Figure 26 pH-dependent activity profile and substrate specificity of consensus phytase-10 and its variants thermo-Q50T and thermo-Q50T-K91A. The phytase activity was determined using the standard assay in appropriate buffers (see Example 11) at different pH-values. Graph a) shows the pH-dependent activity profile of consensus phytase-10 (□), consensus phytase-10-thermo-Q50T (•), and consensus phytase-10-thermo-Q50T-K91A (∧). Graph b) shows the corresponding substrate specificity tested by replacement of phytate by the indicated compounds in the standard assay; open bars, consensus phytase-10 (grey bars, consensus phytase-10-thermo-Q50T; dark bars, consensus phytase-10-thermo-Q50T-K91A). The numbers correspond to the following compounds: 1, phytate; 2, *p*-nitrophenyl phosphate; 3, phenyl phosphate; 4, fructose-1,6-bisphosphate; 5, fructose-6-phosphate; 6, glucose-6-phosphate; 7, ribose-5-phosphate; 8, DL-glycerol-3-phosphate; 9, glycerol-2-phosphate; 10, 3-phosphoglycerate; 11, phosphoenolpyruvate; 12, AMP; 13, ADP; 14, ATP.

Figure 27 pH-dependent activity profile and substrate specificity of consensus phytase-1-thermo[8]-Q50T and of consensus phytase-1-thermo[8]-Q50T-K91A. The phytase activity was determined using the standard assay in appropriate buffers (see Example 11) at different pH-values. Graph a) shows the pH-dependent activity profile of the Q50T- (■) and the Q50T-K91A-variant (•). Graph b) shows the corresponding substrate specificities tested by replacement of phytate by the indicated compounds in the standard assay (open bars, consensus phytase-1-thermo[8]-Q50T; filled bars, consensus phytase-1-thermo[8]-Q50T-K91A.). The substrates are listed in the legend of Figure 26.

Figure 28 Differential scanning calorimetry (DSC) of consensus phytase-1-thermo[8]-Q50T and consensus phytase-1-thermo[8]-Q50T-K91A. The protein samples were concentrated to ca. 50-60 mg/ml and extensively dialyzed against 10 mM sodium acetate, pH 5.0. A constant heating rate of 10 °C/min was applied up to 95 °C. DSC of consensus phytase-1-thermo[8]-Q50T (upper graph) showed a melting temperature of 84.7 °C, while the melting point of consensus phytase-1-thermo[8]-Q50T-K91A was found at 85.7 °C.

Figure 29 Comparison of the temperature optimum between consensus phytase-1, consensus phytase-1-thermo[3] and consensus phytase-1-thermo[8]. For the determination of the temperature optimum, the phytase standard assay was performed at a series of temperatures between 37 and 86 °C. Purified protein from the supernatant of transformed *S. cerevisiae* strains was used for the determination. ○ , consensus phytase-1; □, consensus phytase-1-thermo[3]; ▲, consensus phytase 1-thermo[8].

Figure 30 Comparison of the pH-dependent activity profile and substrate specificity of consensus phytase-1, consensus phytase-7, and of the phytase from *A. niger* NRRL 3135. The phytase activity was determined using the standard assay in appropriate buffers (see Example 11) at different pH-values. Graph a) shows the pH-dependent activity profile of consensus phytase-1 (■), the phytase from *A. niger* NRRL 3135 (○), and of consensus phytase-7 (▲). Graph b) shows the corresponding substrate specificity tested by replacement of phytate by the indicated compounds in the standard assay (black bars, *A. niger* NRRL 3135 phytase; grey bars, consensus phytase-1, dashed bars, consensus phytase-7). The substrates are listed in the legend of Figure 26.

Figure 31 Differential scanning calorimetry (DSC) of the phytase from A. fumigatus ATCC 13073 and of its stabilized α-mutant, which contains the following amino acid exchanges F55Y, V100I, F114Y, A243L, S265P, N294D. The protein samples were concentrated to ca. 50-60 mg/ml and extensively dialyzed against 10 mM sodium ace-

tate, pH 5.0. A constant heating rate of 10 °C/min was applied up to 95 °C. DSC of consensus *A. fumigatus* 13073 phytase (upper graph) revealed a melting temperature of 62.5 °C, while the melting point of the α-mutant was found at 67.0 °C

Figure 32 Comparison of the temperature optimum of *A. fumigatus* 13073 wild-type, its *A. fumigatus* α-mutant, and a further stabilized α-mutant (E59A-S126N-R329H-S364T-G404A). For the determination of the temperature optimum, the phytase standard assay was performed at a series of temperatures between 37 and 75 °C. The diluted supernatant of transformed *S. cerevisiae* strains was used for the determination. The other components of the supernatant showed no influence on the determination of the temperature optimum. ○ , *A. fumigatus* ATCC 13073 phytase; ▲, *A. fumigatus* ATCC 13073 α-mutant; □, *A. fumigatus* ATCC 13073 alpha-mutant-(E59A-S126N-R329H-S364T-G404A)-Q27T; ■ , *A. fumigatus* ATCC 13073 α-mutant-(E59A-S126N-R329H-S364T-G404A)-Q27T-K68A. Q27T and K68A corresponds to consensus phytase-1 Q50T and K91A, respectively.

Figure 33 Amino acid sequence of consensus phytase 12 (consphy12) which contains a number of active site residues transferred from the "basidio" consensus sequence to consensus phytase-10-thermo-Q50T-K91A.

Example 1

a) Materials

[0032] Phytic acid (dodecasodium salt) and polyethylene glycols, polyols, sodium dicarboxylates, sodium periodate, adipic acid dihydrazide and other additives were purchased from commercial suppliers. All other chemicals were at least of analytical grade. Five-ml HiTrap desalting columns were obtained from Pharmacia. SDS-PAGE gels (4-12% NuPAGE Bis-Tris Pre-Cast) and buffers were delivered by NOVEX.

b) Expression and purification of phytases

[0033] *A. fumigatus*, *A. terreus* CBS phytase and consensus phytase were overexpressed in *Hansenula polymorpha*. *A. niger* and *A. nidulans* phytase were overexpressed in *A. niger* Cloning, purification and characterization of these phytases was previously described by Pasamontes *et al* [Appl. Environ. Microbiol. (1997), 63, p. 1696-1700]. Construction, cloning and purification of consensus phytase were performed according to European Patent Application Publication No. 897 985. Non-formulated consensus phytase had an increased thermal stability of up to 70 °C and, due to an amino acid exchange (L at position 50 for Q), a three-fold higher specific activity compared to *A. fumigatus* phytase.

c) Phytase activity assay

[0034] For the determination of thermostability the enzymatic activity measurements with phytic acid were done at different temperatures by diluting the purified enzymes to 0.05 U/ml (activities measured at 37 °C) in 0.2 M sodium acetate, pH 5.0 (+/- additives in % w/w). An aliquot of the protein solution (250 µl) was preincubated for 5 mm at the desired temperature, followed by addition of an equal volume of a solution containing 1% phytic acid in 0.2 M sodium acetate, pH 5.0 (preincubated as a 10 ml aliquot for 10 mm at the same temperature). After incubation of the sample for 15 mm at the desired temperature (e.g. at 60 or 65 °C for the screening of additive effects), the reaction was stopped by addition of
0.5 ml 15% trichloroacetic acid. Determination of liberated inorganic phosphate was performed by standard methods.

d) Evaluation of thermostabilizing additives

[0035] In general, the polyols have been dissolved at a concentration of 25 or 50% (w/w) in 0.2 M sodium acetate, pH 5.0. PEGs have been dissolved at a concentration of 50% with the exception of PEGs with a molecular weight of 4000, 8000 and 10000 which were used at a concentration of 25%. For the screening of PEGs and other polyols, the preincubation and reaction temperature was chosen as 60 °C for *A. nidulans* and *A. terreus* CBS phytase, 65 °C for *A. fumigatus* and *A. niger* phytase and 75 °C for consensus phytase.

[0036] Disodium malonate, succinate and glutarate were dissolved at concentrations of 5, 10 and 25% and phytase activity was measured after preincubation of enzyme plus additive and substrate (see above) at the following temperatures: 37, 45, 50, 55, 60, 65, 70, 75, 80, and 85 °C. In the same way, the temperature dependence of the activity of different phytases in the presence of 25% xylitol and ribitol was tested. It should be noted that the concentration of the additives was reduced by half after substrate addition.

e) Crosslinking f carbohydrate chains

[0037] Crosslinking of phytase carbohydrate chains was performed as described for invertase by Cesi et al. [Studies in Organic Chemistry 47: Stability and Stabilization of Enzymes, Proceedings of an International Symposium held in Maastricht, The Netherlands, 1992, Elsevier Science Publications B.V., Amsterdam, The Netherlands]. Phytase samples (5 mg protein/ml) were incubated for 2 h at 30 °C in the presence of different concentrations (0, 5, 10, 20, 30, 40 and 50 mM) of sodium periodate in 0.2 M sodium acetate, pH 5.0, and stored at 4 °C overnight. Each sample was desalted on a 5-ml HiTrap desalting column (Pharmacia) connected to an ÄktaExplorer system (Pharmacia), using 0.2 M sodium acetate, pH 5.0, as elution buffer. Crosslinking was achieved by adding 100 µl of 0.5 M adipic acid dihydrazide dissolved in 0.2 M sodium acetate, pH 5.0, to 900 µl of the desalted oxidation products. Phytase activity measurements and gel electrophoresis of the samples were performed after both the oxidation and crosslinking steps.

f) High-shear granulation of thermostabilized phytases

[0038] 100-250 ml of a phytase solution (in total 2500 - 5000 units of crosslinked or non-crosslinked phytase) were added to 1 kg of a dry mixture of 5-10% calcium lignosulfonate (Borregard, Norway), 5-20% silica (Sipernat 50S, Degussa, Germany), 0-20% thermostabilizing agent and gipsum. During the high-shear granulation process water was added until granulates with desired properties were formed. The granulates were dried in a fluid bed dryer for 15 mm at 45 °C and subsequently fat coated with natural palm fat (Palm 46, Florin, Basel, Switzerland).

g) Pelleting stability of thermostabilized dry and liquid phytase formulations

[0039] Thermostabilized dry or liquid formulations of phytases (as mentioned above) were mixed with feed and subsequently pelleted under steam conditioning at 85 °C. The pelleting stability of phytase was determined by measurement of the phytase activity both in the mash before pelleting and in the delivered pellets.

Example 2

[0040] Investigations of the temperature dependence of activity of different fungal phytases as described in Example 1 revealed activity maxima at the following temperatures: 55 °C for *A. fumigatus phytase* and *A. niger* phytase, 45 °C for *A. terrreus* CBS phytase and *A. nidulans* phytase, and 65 °C for consensus phytase. A temperature 10-15 °C above the determined temperature maximum was chosen as screening point for studying the effects of polyols, polyethylene glycols, dicarboxylates, carboxymethylcellulose and sodium alginate on the thermostability of phytases.

a) Addition of polyethylene glycols of different molecular weights

[0041] The addition of 50% or 25% (25% and 12.5% final concentration during the reaction period) polyethylene glycol enhanced the specific activity of *A. fumigatus* phytase measured at 65 °C in a molecular weight-dependent fashion, with a maximum being observed with PEG 1450 (specific activity 80 U*(mg protein)$^{-1}$) and considerable activities also with PEG 1000 (50 U*(mg protein)$^{-1}$) and PEG 3350 (42 U*(mg protein)$^{-1}$). The results of this experiment are summarized in Figure 2.

[0042] PEGs with molecular weights of 600, 1000, 1450, 3350 and 4000 Da showed similar effects on the other phytases tested. The results of this experiment are shown in Figure 3.

b) Addition of polyols

[0043] The polyols ribitol, xylitol (C_5 sugars) and sorbitol (C_6 sugar) in concentrations of 25 and 50% significantly improved the thermostability of *A. fumigatus* phytase. This is shown in Figure 4.

[0044] Erythritol, mannitol, mannoheptulose and mannoheptose were not soluble in 0.2 M sodium acetate, pH 5.0, at a concentration of 50% (w/w) and, therefore, only the 25% values are shown. The specific activities measured at 65 °C were 11, 21 and 11 U*(mg protein)$^{-1}$ in the presence of 25% ribitol, xylitol and sorbitol, and 51, 90 and 74 U*(mg protein)$^{-1}$ in the presence of 50% solutions of ribitol, xylitol and sorbitol, respectively.

[0045] Polyols containing more than 6 or less than 5 carbon atoms such as glycerol (C_3 sugar), erythritol (C_4 sugar), mannoheptose and mannoheptulose (C_7 sugars) showed an inferior effect on the thermostabilization of *A. fumigatus* phytase.

[0046] Xylitol at a concentration of 50% also increased the temperature optimum of *A. nidulans*, *A. terreus* CBS, *A. niger* and consensus phytase by 10-15 °C. The results are shown in Figure 5.

c) Addition of dicarboxylic acids

[0047] Malonate, succinate and glutarate at a concentration of 25% (12.5% final concentration in the activity assay) resulted in a significant increase in *A. fumigatus* phytase thermostability with considerable activity still being detected at 70 °C for malonate and at 65 °C for succinate and glutarate. The results are shown in Figure 6.

[0048] In addition, dicarboxylates stimulated *A. fumigatus* phytase activity measured at 37 °C, with an approximately 4-fold increase in phytase activity in the case of malonate, a 2-fold increase for succinate and minor effects for glutarate. Investigation of different concentrations (5, 10 and 25%) of malonate showed that thermostabilization of *A. fumigatus* phytase is concentration-dependent whereas stimulation of enzymatic activity, at least in this concentration range, is not This is shown in Figure 7.

[0049] In contrast to these findings, different concentrations of sodium acetate (5, 10 and 25%), a monocarboxylic acid. caused a 2-fold increase in specific activity of *A. fumigatus* phrase at 37 °C, but had only minor effects on the thermal stability of the protein. This can be seen in Figure 8.

[0050] Disodium malonate and succinate generally increased the thermostability of *A. nidulans*, *A. terreus* CBS, *A. niger* and consensus phytase by 5-15 °C. On the other hand, stimulation of phytase activity was only observed for *A. nidulans* and *A. fumigatus* phytase, both having a rather low specific activity, but not for *A. terreus* CBS, *A. niger* and consensus phytase. This is demonstrated in Figures 9 and 10.

d) Effect of crosslinking

[0051] In a preliminary experiment, *A. fumigatus* phytase monomers were crosslinked by incubation with glutaraldehyde. The resulting thermostabilization measured at 60 °C reached a maximum after 1 hr reaction time but led to activity loss (measured at 37 °C). In a further set of experiments, *A. fumigatus* phytase monomers were crosslinked via their carbohydrate chains. This type of crosslinking was achieved with only minor loss of specific activity (< 10%) and resulted in the formation of oligomeric forms at sodium periodate concentrations above 15 mM. This can be seen from Figure 11.

[0052] The extent of thermostabilization was dependent on periodate concentration and reached a maximum at 50 mM where high specific activities were observed up to 75 °C (see Figure 12). A pronounced effect of phytase oligomerization on thermostability was also observed for consensus phytase crosslinked via its carbohydrate chains. This can be seen from Figure 12.

[0053] In the present work, we focused our efforts on the thermostabilization effects of low-M_r additives - which are highly recommended for stabilization of industrial enzymes - and of chemical modification - even though this latter approach is commonly regarded as less attractive for technical and economical reasons.

[0054] We have found thermostabilization by C_5 sugars for a range of different phytases expressed in filamentous fungi (*A. niger*) or yeasts (*Hansenula polymorpha*). The increase in thermostability varied to some extent between the different phytases, but was around 10 °C. The effect of PEGs was molecular weight-dependent. The optimal thermostabilization of all phytases was obtained with PEGs having a molecular weight between 1000 and 3350 Da.

[0055] Sodium acetate, a monocarboxylic acid and main component of the standard phytase activity assay, caused a concentration-dependent increase in *A. fumigatus* phytase activity, but had no effect on phytase thermostability. Therefore, carboxylate groups might be responsible for the activity modulation whereas bifunctional dicarboxylates possibly stabilize phytases by ionic interactions.

Example 3

Design of the amino acid sequence of consensus phytase-1

Alignment of the amino acid sequences

[0056] The alignment was calculated using the program PILEUP from the Sequence Analysis Package Release 9.0 (Devereux *et al.*, 1984) with the standard parameter (gap creation penalty 12, gap extension penalty 4). The location of the gaps was refined using a text editor. Table 1 shows the sequences (see Figure 13) without the signal sequence that were used for the performance of the alignment starting with the amino acid (aa) as mentioned in Table 1.

Table 1

Origin and vote weight of the phytase amino acid sequences used for the design of consensus phytase-1

-*phyA* from *Aspergillus terreus* 9A-1, aa 27, vote weight 0.5 (Mitchell *et al.*, 1997)

Table 1 (continued)

Origin and vot weight of th phytase amino acid sequences used for the design of consensus phytase-1
-*phyA* from *Aspergillus terreus* cbs116.46, aa 27, vote weight 0.5 (van Loon *et al.*, 1998)
- *phyA* from *Aspergillus niger* var. *awamori*, aa 27, vote weight 0.33 (Piddington *et al.*, 1993)
-*phyA* from *Aspergillus niger* T213, aa 27, vote weight 0.33
- *phyA* from *Aspergillus niger* strain NRRL3135, aa 27, vote weight 0.33 (van Hartingsveldt *et al.*, 1993)
- *phyA* from *Aspergillus fumigatus* ATCC 13073, aa 26, vote weight 0.2 (Pasamontes *et al.*, 1997)
-*phyA* from *Aspergillus fumigatus* ATCC 32722, aa 26, vote weight 0.2 (van Loon *et al.*, 1998)
- *phyA* from *Aspergillus fumigatus* ATCC 58128, aa 26, vote weight 0.2 (van Loon *et al.*, 1998)
- *phyA* from *Aspergillus fumigatus* ATCC 26906, aa 26, vote weight 0.2 (van Loon *et al.*, 1998)
- *phyA* from *Aspergillus fumigatus* ATCC 32239, aa 30, vote weight 0.2 (van Loon *et al.*, 1998)
-*phyA* from *Emericella nidulans* , aa 25, vote weight 1.0, Pasamontes *et al.*, 1997a)
- *phyA* from *Talaromyces rhermophilus* ATCC 20186, aa 24, vote weight 1.0 (Pasamontes *et al.*, 1997a)
-*phyA* from *Myceliophthora thermophila*, aa 19, vote weight 1.0 (Mitchell *et al.*, 1997)

Calculation of the amino acid sequence of consensus phytase-1

[0057] Using the refined alignment as input, the consensus sequence was calculated by the program PRETTY from the Sequence Analysis Package Release 9.0 (Devereux *et al.*, 1984). PRETTY prints sequences with their columns aligned and can display a consensus sequence for an alignment. A vote weight that pays regard to the similarity between the amino acid sequences of the phytases aligned was assigned to all sequences. The vote weight was set such as the combined impact of all phytases from one sequence subgroup (same species, but from different strains), e. g. the amino acid sequences of all phytases from *A. fumigatus*, on the election was set one, that means that each sequence contributes with a value of 1 divided by the number of strain sequences (see Table 1). By this means, it was possible to prevent that very similar amino acid sequences, e. g. of the phytases from different *A. fumigatus* strains, dominate the calculated consensus sequence.

[0058] The program PRETTY was started with the following parameters: The plurality defining the number of votes below which there is no consensus was set on 2.0. The threshold, which determines the scoring matrix value below which an amino acid residue may not vote for a coalition of residues, was set on 2. PRETTY used the PrettyPep.Cmp consensus scoring matrix for peptides.

[0059] Ten positions of the alignment (position 46, 66, 82, 138, 162, 236, 276, 279, 280, 308; Figure 13), for which the program was not able to determine a consensus residue, were filled by hand according to the following rules: if a most frequent residue existed, this residue was chosen (138, 236, 280); if a prevalent group of similar or phylogenetically equivalent residues occurred, the most frequent or, if not available, one residues of this group was selected (46, 66, 82, 162, 276, 308). If there was either a prevalent residue nor a prevalent group, one of the occurring residues was chosen according to common assumption on their influence on the protein stability (279). Eight other positions (132, 170, 204, 211, 275, 317, 384, 447; Figure 13) were not filled with the amino acid residue selected by the program but normally with amino acids that occur with the same frequency as the residues that were chosen by the program. In most cases, the slight underrating of the three *A. niger* sequences (sum of the vote weights: 0.99) was eliminated by this corrections.

Conversion of the consensus phytase-1 amino acid sequence to a DNA sequence

[0060] The first 26 amino acid residues of *A. terreus* cbs116.46 phrase were used as signal peptide and, therefore, fused to the N-terminus of all consensus phrases. For this stretch, we used a special method to calculate the corresponding DNA sequence. Purvis et al (1987) proposed that the incorporation of rare codons in a gene has an influence on the folding efficiency of the protein. Therefore, at least the distribution of rare codons in the signal sequence of *A. terreus* cbs116.46, which was used for the consensus phrase and which is very important for secretion of the protein, but converted into the *S. cerevisiae* codon usage, was transferred into the new signal sequence generated for expression in *S. cerevisiae*. For the remaining parts of the protein, we used the codon frequency table of highly expressed *S. cerevisiae* genes, obtained from the GCG program package, to translate the calculated amino acid sequence into a DNA sequence.

[0061] The resulting sequence of the *fcp* gene is shown in Figure 14.

Constructi n and cloning of the consensus phytase-1 gene

[0062] The calculated DNA sequence of consensus phytase-1 (*fcp*) was divided into oligonucleotides of 85 bp, alternately using the sequence of the sense and the anti-sense strand. Every oligonucleotide overlaps 20 bp with its previous and its following oligonucleotide of the opposite strand. The location of all primers, purchased by Microsynth, Balgach (Switzerland) and obtained in a PAGE-purified form, is indicated in Figure 14.

PCR-Reactions

[0063] In three PCR reactions, the synthesized oligonucleotides were composed to the entire gene. For the PCR, the High Fidelity Kit from Boehringer Mannheim (Boehringer Mannheim, Mannheim, Germany) and the thermo cycler The Protokol™ from AMS Biotechnology (Europe) Ltd. (Lugano, Switzerland) was used.

[0064] Oligonucleotide CP-1 to CP-10 (Mix 1, Figure 14) were mixed to a concentration of 0.2 pMol/µl of each oligonucleotide. A second oligonucleotide mixture (Mix 2) was prepared with CP-9 to CP-22 (0.2 pMol/µl of each oligonucleotide). Additionally, four short primers were used in the PCR reactions:

CP-a: *Eco* RI

5'-TATAT*GAATTC*ATGGGCGTGTTCGTC-3'

CP-b:

5'-TGAAAAGTTCATTGAAGGTTTC-3'

CP-c:

5'-TCTTCGAAAGCAGTACAAGTAC-3'

CP-e: *Eco* RI

5'-TATAT*GAATTC*TTAAGCGAAAC-3'

PCR reaction α: 10 µl Mix 1 (2.0 pmol of each oligonucleotide)

2 µl nucleotides (10 mM each nucleotide)
2 µl primer CP-a (10 pmol/µl)
2 µl primer CP-c (10 pmol/µl)
10,0 µl PCR buffer
0.75 µl polymerase mixture
73.25 µl H_2O

PCR reaction *b*: 10 µl Mix 2 (2.0 pmol of each oligonucleotide)

2 µl nucleotides (10 mM each nucleotide)
2 µl primer CP-b (10 pmol/µl)
2 µl primer CP-e (10 pmol/µl)
10,0 µl PCR buffer
0.75 µl polymerase mixture (2.6 U)

73.25 µl H_2O

Reaction conditions for PCR reaction *a* and *b*:

step 1	2 min - 45°C
step 2	30 sec - 72°C
step 3	30 sec - 94°C
step 4	30 sec - 52°C
step 5	1 min - 72°C

[0065] Step 3 to 5 were repeated 40-times.

[0066] The PCR products (670 and 905 bp) were purified by an agarose gel electrophoresis (0.9% agarose) and a following gel extraction (QIAEX II Gel Extraction Kit, Qiagen, Hilden, Germany). The purified DNA fragments were used for the PCR reaction *c*.

PCR reaction *c*: 6 µl PCR product of reaction a (≈ 50 ng)

6 µl PCR product of reaction b (≈ 50 ng)
2 µl primer CP-a (10 pmol/µl)
2 µl primer CP-e (10 pmol/µl)
10.0 µl PCR buffer
0.75 µl polymerase mixture (2.6 U)
73.25 µl H_2O

Reaction conditions for PCR reaction *c*:

step 1	2 mm - 94°C
step 2	30 sec - 94°C
step 3	30 sec - 55°C
step 4	1 mm - 72°C

[0067] Step 2 to 4 were repeated 31-times.

[0068] The resulting PCR product (1.4 kb) was purified as mentioned above, digested with *Eco* RI, and ligated in an *Eco* RI-digested and dephosphorylated pBsk(-)-vector (Stratagene, La Jolla, CA, USA). 1 µl of the ligation mixture was used to transform *E. coli* XL-1 competent cells (Stratagene, La Jolla, CA, USA). All standard procedures were carried out as described by Sambrook *et al.* (1987). The DNA sequence of the constructed consensus phytase gene (*fcp*, Figure 14) was controlled by sequencing as known in the art.

Example 4

Design of an improved consensus phytase (consensus phytase-10) amino acid sequence

[0069] The alignments used for the design of consensus phytase-10 were calculated using the program PILEUP from the Sequence Analysis Package Release 9.0 (Devereux *et al.*, 1984) with the standard parameter (gap creation penalty 12, gap extension penalty 4). The location of the gaps was refined using a text editor.

[0070] The following sequences were used for the alignment of the *Basiodiomycetes* phytases starting with the amino acid (aa) mentioned in Table 2:

Table 2

Origin and vote weight of five *Basidiomycetes* phytases used for the calculation of the corresponding amino acid consensus sequence (basidio)

- *phyA1* from *Paxillus involutus* NN005693, aa 21, vote weight 0.5 (WO 98/28409)
- *phyA2* from *Paxillus involutus* NN005693, aa 21, vote weight 0.5 (WO 98/28409)
- *phyA* from *Trametes pubescens* NN9343, aa 24, vote weight 1.0 (WO 98/28409)
- *phyA* from *Agrocybe pediades* NN009289, aa 19, vote weight 1.0 (WO 98/28409)
- *phyA* from *Peniophora lycii* NN006113, aa 21, vote weight 1.0 (WO 98/28409)

[0071] The alignment is shown in Figure 3.

[0072] In Table 3 the genes, which were used for the performance of the final alignment, are arranged. The first amino acid (aa) of the sequence which is used in the alignment is mentioned behind the organism designation.

Table 3

Origin and vote weight of the phytase sequences used for the design of consensus phytase 10

- *phyA* from *Aspergillus terreus* 9A-1, aa 27, vote weight 0.5 (Mitchell *et al.*, 1997)
- *phyA* from *Aspergillus terreus* cbs116.46, aa 27, vote weight 0.5 (van Loon *et al.*, 1998)
- *phyA* from *Aspergillus niger* var. *awamori*, aa 27, vote weight 0.5 (Piddington *et al.*, 1993)
- *phyA* from *Aspergillus niger* strain NRRL3135, aa 27, vote weight 0.5 (van Hartingsveldt *et al.*, 1993)
- *phyA* from *Aspergillus fumigatus* ATCC 13073, aa 26, vote weight 0.2 (Pasamontes *et al.*, 1997)
- *phyA* from *Aspergillus fumigatus* ATCC 32722, aa 26, vote weight 0.2 (van Loon *et al.*, 1998)
- *phyA* from *Aspergillus fumigatus* ATCC 58128, aa 26, vote weight 0.2 (van Loon *et al.*, 1998)
- *phyA* from *Aspergillus fumigatus* ATCC 26906, aa 26, vote weight 0.2 (van Loon *et al.*, 1998)
- *phyA* from *Aspergillus fumigatus* ATCC 32239, aa 30, vote weight 0.2 (van Loon *et al.*, 1998)
- *phyA* from *Emericella nidulans* , aa 25, vote weight 1.0, Pasamontes *et al.*, 1997a)
- *phyA* from *Talaromyces thermophilus* ATCC 20186, aa 24, vote weight 1.0 (Pasamontes *et al.*, 1997a)
- *phyA* from *Myceliophthora thermophila*, aa 19, vote weight 1.0 (Mitchell *et al.*, 1997)
- *phyA* from *Thermomyces lanuginosa*, aa 36, vote weight 1.0 (Berka *et al.*, 1998)
- Consensus sequence of five *Basidiomycetes* phytases, vote weight 1.0 (Basidio, Figure 15)

[0073] The corresponding alignment is shown in Figure 16.

Calculation of the amino acid sequence of consensus-10

[0074] To improve the alignment, we added the original consensus sequence of five phytases from four different *Basidiomycetes*, called Basidio, still containing the undefined sequence positions (see Figure 15), nearly all phytase sequences used for calculation of the original consensus phytase and one new phytase sequence from the *Ascomycete Thermomyces lanuginosa* to a larger alignment. Using the consensus sequence of the basidiomycetal phytase sequences, does not pay regard to the diversity among the five amino acid sequences, but pays regard to the common and different amino acid residues between the phytases from the *Ascomycetes* and the *Basidiomycetes*.

[0075] We set plurality on 2.0 and threshold on 3. The used vote weight are listed in Table 3. The alignment and the corresponding consensus sequence is presented in Figure 16. The new consensus phytase sequence has 32 different amino acids in comparison to the original consensus phytase. Positions for which the program PRETTY was not able to calculate a consensus amino acid residue were filled according to rules mentioned in Example 3. None of the residues suggested by the program was replaced.

[0076] Furthermore, we included all *Basidiomycetes* phytases as single amino acid sequences but assigning a vote weight of 0.2 in the alignment. The corresponding alignment is shown in Figure 18. The calculated consensus amino acid sequence (consensus phytase-11) has the following differences to the sequence of consensus phytase-10. Letter X means that the program was not able to calculate a consensus amino acid; the amino acid in parenthesis corresponds to the amino acid finally included into the consensus phytase-10.

D35X, X(K)69K, X(E)100E, A101R, Q134N, X(K)153N, X(H)190H, X(A)204S, X(E)220D, E222T, V227A, X(R)271R, H287A, X(D)288D, X(K)379K, X(I)389I, E390X, X(E)415E, X(A)416A, X(R)446L, E463A, whereas the numbering is as in Fig. 17.

[0077] We also checked single amino acid replacements suggested by the improved consensus sequences 10 and 11 on their influence on the stability of the original consensus phytase. The approach is described in example 5.

Conversion of consensus phytase-10 amino acid sequence to a DNA sequence

[0078] The first 26 amino acid residues of *A. terreus* cbs116.46 phytase were used as signal peptide and, therefore, fused to the *N*-terminus of consensus phytase-10. The used procedure is further described in Example 3.

[0079] The resulting sequence of the *fcp*10 gene is shown in Figure 17.

Construction and cloning of the consensus phytase-10 gene (*fcp*10)

[0080] The calculated DNA sequence of *fcp*10 was divided into oligonucleotides of 85 bp, alternately using the sequence of the sense and the anti-sense strand. Every oligonucleotide overlaps 20 bp with its previous and its following oligonucleotide of the opposite strand. The location of all primers, purchased by Microsynth, Balgach (Switzerland) and obtained in a PAGE-purified form, is indicated in Figure 17.

PCR-Reactions

[0081] In three PCR reactions, the synthesized oligonucleotides were composed to the entire gene. For the PCR, the High Fidelity Kit from Boehringer Mannheim (Boehringer Mannheim, Mannheim, Germany) and the thermo cycler The Protokol™ from AMS Biotechnology (Europe) Ltd. (Lugano, Switzerland) was used. The following oligonucleotides were used in a concentration of 0.2 pMol/µl.

Mix 1.10: CP-1, CP-2, CP-3.10, CP-4.10, CP-5.10, CP-6, CP-7.10, CP-8.10, CP-9.10, CP-10.10

Mix 2.10: CP-9.10, CP-11.10, CP-12.10, CP-13.10, CP-14.10, CP-15.10, CP-16.10, CP-17.10, CP18.10, CP-19.10, CP-20.10, CP-21.10, CP-22.10

[0082] The newly synthesized oligonucleotides are marked by number 10. The phytase contains the following 32 exchanges, which are underlined in Figure 17, in comparison to the original consensus phytase: Y54F, E58A, D69K, D70G, A94K, N134Q, I158V, S187A, Q188N, D197N, S204A, T214L, D220E, L234V, A238P, D246H, T251N, Y259N, E267D, E277Q, A283D, R291I, A320V, R329H, S364T, I366V, A379K, S396A, G404A, Q415E, A437G, A463E.

[0083] Four short PCR primer were used for the assembling of the oligonucleotides:

CP-a: *Eco* RI

5'-TATAT*GAATTC*ATGGGCGTGTTCGTC-3'

CP-b:

5'-TGAAAAGTTCATTGAAGGTTTC-3'

CP-c.10:

5'-TCTTCGAAAGCAGTACACAAAC-3'

CP-e: *Eco* RI

5'-TATAT*GAATTC*TTAAGCGAAAC-3'

PCR reaction *a*: 10 µl Mix 1.10 (2.0 pmol of each oligonucleotide)

2 µl nucleotides (10 mM each nucleotide)
2 µl primer CP-a (10 pmol/ml)
2 µl primer CP-c.10 (10 pmol/ml)
10,0 µl PCR buffer
0.75 µl polymerase mixture
73.25 µl H_2O

PCR reaction *b*: 10 µl Mix 2.10 (2.0 pmol of each oligonucleotide)

2 µl nucleotides (10 mM each nucleotide)
2 µl primer CP-b (10 pmol/ml)
2 µl primer CP-e (10 pmol/ml)
10,0 µl PCR buffer
0.75 µl polymerase mixture (2.6 U)
73.25 µl H_2O

Reaction conditions for PCR reaction *a* and *b*:

step 1	2 min - 45°C
step 2	30 sec - 72 °C
step 3	30 sec - 94 °C
step 4	30 sec - 52 °C
step 5	1 min - 72°C

[0084] Step 3 to 5 were repeated 40-times.

[0085] The PCR products (670 and 905 bp) were purified by an agarose gel electrophoresis (0.9% agarose) and a following gel extraction (QIAEX II Gel Extraction Kit, Qiagen, Hilden, Germany). The purified DNA fragments were used for the PCR reaction *c*.

PCR reaction *c*: 6 µl PCR product of reaction a ≈50 ng)

6 µl PCR product of reaction b ≈50 ng)
2 µl primer CP-a (10 pmol/ml)
2 µl primer CP-e (10 pmol/ml)
10,0 µl PCR buffer
0.75 µl polymerase mixture (2.6 U) 73.25 µl H_2O

Reaction conditions for PCR reaction *c*:

step 1	2 min - 94°C
step 2	30 sec - 94 °C
step 3	30 sec - 55 °C
step 4	1 min - 72 °C

[0086] Step 2 to 4 were repeated 31-times.

[0087] The resulting PCR product (1.4 kb) was purified as mentioned above, digested with *Eco* RI, and ligated in an *Eco* RI-digested and dephosphorylated pBsk(-)-vector (Stratagene, La Jolla, CA, USA). 1 µl of the ligation mixture was used to transform *E. coli* XL-1 competent cells (Stratagene, La Jolla, CA, USA). All standard procedures were carried out as described by Sambrook *et al.* (1987). The DNA sequence of the constructed gene (*fcp10*) was checked by sequencing as known in the art.

Example 5

Increasing the thermostability of consensus phytase-1 by introduction of single mutations suggested by the amino acid sequence of consensus phytase-10 and consensus phytase-11

[0088] In order to increase the thermostability of homologous genes, it is also possible to test the stability effect of each differing amino acid residue between the protein of interest and the calculated consensus sequence and to combine all stabilizing mutations into the protein of interest. We used the consensus phytase as protein of interest and tested the effect on the protein stability of 34 amino acid residues, differing to consensus phytase 10 and/or 11 as single mutations.

[0089] To construct muteins for expression in *A. niger, S. cerevisiae*, or *H. polymorpha*, the corresponding expression plasmid containing the consensus phytase gene was used as template for site-directed mutagenesis (see Example 8 - 10). Mutations were introduced using the "quick exchange™ site-directed mutagenesis kit" from Stratagene (La Jolla, CA, USA) following the manufacturer's protocol and using the corresponding primers. All mutations made and their corresponding primers are summarized in Table 4. Plasmids harboring the desired mutation were identified by DNA sequence analysis as known in the art.

Table 4: Primers used for site-directed mutagenesis of consensus phytase

(Exchanged bases are highlighted in bold. The introduction of a restriction site is marked above the sequence. When a restriction site is written in parenthesis, the mentioned site was destroyed by introduction of the mutation.)

mutation	Primer set
	Kpn I-
Q50T	5'-CACTTGTGG*GGTACC*TACTCTCCATACTTCTC-3'
	5'-GAGAAGTATGGAGAGTA*GGTACC*CCACAAGTG-3'
Y54F	5'-GGTCAATACTCTCCATTCTTCTCTTTGGAAG-3'
	5'-CTTCCAAAGAGAAGAATGGAGAGTATTGACC-3'
E58A	5'-CATACTTCTCTTTGGCAGACGAATCTGC-3'
	5'-GCAGATTCGTCTGCCAAAGAGAAGTATG-3'
	Aat II
D69K	5'-CTCCA*GACGTC*CCAAAGGACTGTAGAGTTAC-3'
	5'-GTAACTCTACAGTCCTTTGG*GACGTC*TGGAG-3'
	Aat II
D70G	5'-CTCCA*GACGTC*CCAGACGGCTGTAGAGTTAC-3'
	5'-GTAACTCTACAGCCGTCTGG*GACGTC*TGGAG-3'
K91A	5'-GATACCCAACTTCTTCTGCGTCTAAGGCTTACTCTG-3'
	5'-CAGAGTAAGCCTTAGACGCAGAAGAAGTTGGGTATC-3'

ScaI

A94K 5'-CTTCTAAGTCTAAG**A***AGTACT*CTGCTTTG-3'

5'-CAAAGCAG*AGTACT*TCTTAGACTTAGAAG-3'

A101R 5'-GCTTACTCTGCTTTGATTGAA**CG**GATTCAAAAGAACGCTAC-3'

5'-GTAGCGTTCTTTTGAAT**CCG**TTCAATCAAAGCAGAGTAAGC-3'

N134Q 5'-CCATTCGGTGAA**CAG**CAAATGGTTAACTC-3'

5'-GAGTTAACCATTTG**CTG**TTCACCGAATGG-3'

Nru I

K153N 5'-GATACAAGGCT*CTCGCGA*GAAA**C**ATTGTTC –3'

5'-GGAACAAT**G**TTTC*TCGCGA***G**AGCCTTGTATC-3'

Bss HI

I158V 5'-GATTGTTCCATTC**GT***GCGCGC*TTCTGGTTC-3'

5'-GAACCAGAA*GCGCGC*ACGAATGGAACAATC-3'

Bcl I

D197N 5'-CTCCAGTTATT**AACG***TGATCA*TTCCAGAAGG-3'

5'-CCTTCTGGAA*TGATCA*C**GTT**AATAACTGGAG-3'

Apa I

S187A 5'-GGCTGACCCAG***GGCCC***AACCACACCAAGC-3'

5'-GCTTGGTGTGGTT*GGGCCC*CTGGGTCAGCC-3'

Nco I

T214L 5'-CACTTTGGA*CCATGG***T**CTTTGTACTGCTTTCG-3'

5'-CGAAAGCAGTACAAA**G**A*CCATGG*TCCAAAGTG-3'

Avr II

E222T 5'-GCTTTCGAAGACTCTA***CCCTAGG***TGACGACGTTG-3'

5'-CAACGTCGTCA*CCTAGG***G**TAGAGTCTTCGAAAGC-3'

	V227A	5'-GGTGACGACGCTGAAGCTAACTTCAC-3'
		5'-GTGAAGTTAGCTTCAGCGTCGTCACC-3'
		Sac II
	L234V	5'-CTAACTTCA*CCGCGG*TGTTCGCTCCAG-3'
		5'-CTGGAGCGAACACCGCGGTGAAGTTAG-3'
	A238P	5'-GCTTTGTTCGCTCCACCTATTAGAGCTAGATTGG-3'
		5'-CCAATCTAGCTCTAATAGGTGGAGCGAACAAAGC-3'
		Hpa I
	T251N	5'-GCCAGGT*GTTAAC*TTGACTGACGAAG-3'
		5'-TTCGTCAGTCAA*GTTAAC*ACCTGGC-3'
		Aat II
	Y259N	5'-GACGAA*GACGTC*GTTAACTTGATGGAC-3'
		5'-GTCCATCAAGTTAAC*GACGTC*TTCGTC-3'
		Asp I
	E267D	5'-GTCCATTC*GACACTGTC*GCTAGAACTT C-3'
		5'-GAAGTTCTAGC*GACAGTGTC*GAATGGAC-3'
	E277Q	5'-CTGACGCTACTCAGCTGTCTCCATTC-3'
		5'-GAATGGAGACAGCTGAGTAGCGTCAG-3'
	A283D	5'-GTCTCCATTCTGTGATTTGTTCACTCAC-3'
		5'-GTGAGTGAACAAATCACAGAATGGAGAC-3'
		Ksp I
	H287A	5'-GCTTTGTTCAC*CGCGG*ACGAATGGAG-3'
		5'-CTCCATTCGT*CCGCGG*TGAACAAAGC-3'

Bam HI

R291I 5'-CACGACGAAT*GGATCC*AATACGACTAC-3'

5'-GTAGTCGTATT*GGATCC*ATTCGTCGTG-3'

Bsi WI

Q292A 5'-GACGAATGGAGA*GCGTACG*ACTACTTG-3'

5'-CAAGTAGT*CGTACG*CTCTCCATTCGTC-3'

Hpa I

A320V 5'-GGTGTTGGTTTC*GTTAAC*GAATTGATTGC-3'

5'-GCAATCAATTC*GTTAAC*GAAACCAACACC-3'

(Bgl II)

R329H 5'-GCTAGATTGACT*CACTCT*CCAGTTCAAG-3'

5'-CTTGAACTGGAGA*GTGAGT*CAATCTAGC-3'

Eco RV

S364T 5'-CTCACGACAACACTAT*GATATC*TATTTTCTTC-3'

5'-GAAGAAAATA*GATATC*ATAGTGTTGTCGTGAG-3'

Nco I

I366V 5'-CGACAACT*CCATGG*TTTCTATTTTCTTCGC-3'

5'-GCGAAGAAAATAGAAA*CCATGG*AGTTGTCG-3'

Kpn I

A379K 5'-GTACAAC*GGTACC*AAGCCATTGTCTAC-3'

5'-GTAGACAATGGCTT*GGTACC*GTTGTAC-3'

S396A 5'-CTGACGGTTACGCTGCTTCTTGGAC-3'

5'-GTCCAAGAAGCAGCGTAACCGTCAG-3'

G404A 5'-CTGTTCCATTCGCTGCTAGAGCTTAC-3'
5'-GTAAGCTCTAGCAGCGAATGGAACAG-3'

Q415E 5'-GATGCAATGTGAAGCTGAAAAGGAACC-3'
5'-GGTTCCTTTTCAGCTTCACATTGCATC-3'

Sal I

A437G 5'-CACGGTTGTGGT*GTCGAC*AAGTTGGG-3'
5'-CCCAACTT*GTCGAC*ACCACAACCGTG-3'

Mun I

A463E 5'-GATCTGGTGG*CAATTG*GGAGGAATGTTTCG-3'
5'-CGAAACATTCCTCC*CAATTG*CCACCAGATC-3'

and accordingly for other mutations.

[0090] The temperature optimum of the purified phytases, expressed in *Saccharomyces cerevisiae* (Example 9), was determined as outlined in Example 11. Table 5 shows the effect on the stability of consensus phytase for each mutation introduced.

Table 5

Stability effect of the individual amino acid replacements in concensus phytase-1					
(+ or - means a positive, respectively, negativ effect on the protein stability up to 1 °C, ++ and -- means a positive, respectively, negative effect on the protein stability between 1 and 3 °C; the number 10 or 11 corresponds to the consensus phytase sequence that suggests the amino acid replacement.)					
stabilizing		neutral		destabilizing	
mutation	effect	mutation	effect	mutation	effect
E58A (10)	+	D69A	±	Y54F (10)	-
D69K (11)	+	D70G (10)	±	V73I	-
D197N (10)	+	N134Q (10)	±	A94K (10)	-
T214L (10)	++	G186H	±	A101R (11)	-
E222T (11)	++	S187A (10)	±	K153N (11)	-
E267D (10)	+	T214V	±	I158V (10)	--
R291I*	+	T251N (10)	±	G203A	--
R329H (10)	+	Y259N (10)	±	G205S	-
S364T (10)	++	A283D (10)	±	A217V	-
A379K (11)	+	A320V (10)	±	V227A (11)	--
G404A (10)	++	K445T	±	L234V (10)	-
		A463E (10)	±	A238P (10)	--
				E277Q (10)	-
				H287A (11)	-
				Q292A (10)	-
				I366V (10)	-
				S396A (10)	--
				Q415E (11)	-
				A437G (10)	--
				E451R	--

*: This amino acid replacement was found in another round of mutations.

[0091] We combined eight positive mutations (E58A, D197N, E267D, R291I, R329H, S364T, A379K, G404A) in the consensus phytase using the primers and the technique mentioned above in this example. Furthermore, the mutations Q50T and K91A were introduced which mainly influence the catalytical characteristics of the phytase (see European Patent Application Publication No. 897 985 as well as Example 11). The DNA and amino acid sequence of the resulting phytase gene (consensus phytase-thermo[8]-Q50T-K91A) is shown in Figure 19. In this way, the temperature optimum and the melting point of the consensus phytase was increased by 7 °C (Figure 27, 28, 29).

[0092] Using the results of Table 5, we further improved the thermostability of consensus phytase 10 by the following back mutations K94A, V158I, and A396S that revealed a strong negative influence on the stability of consensus phytase. The resulting protein is phytase-10-thermo [3]. Furthermore, we introduced the mutations Q50T and K91A which mainly influence the catalytical characteristics of consensus phytase (see patent application EP Publication No. 897 985 as well as Example 11 and Figure 26 and 27). The resulting DNA and amino acid sequence is shown in Figure 20. The optimized phytase showed a 4 °C higher temperature optimum and melting point than consensus phytase 10 (Figure 24 and 25). Furthermore, the phytase has also a strongly increased specific activity with phytate as substrate of 250 U/mg at pH 5.5 (Figure 26).

Example 6

Stabilization of the phytase of *A. fumigatus* ATCC 13073 by replacement of amino acid residues with the corresponding consensus phytase-1 and consensus phytase-10 residues

[0093] At six typical positions where the *A. fumigatus* 13073 is the only or nearly the only phytase in the alignment of Figure 13 that does not contain the corresponding consensus phytase amino acid residue, the non-consensus amino acid residue was replaced by the consensus one. In a first round, the following amino acids were substituted in *A. fumigatus* 13073 phytase, containing the Q27T substitution and the signal sequence of *A. terreus* cbs.116.46 phytase (see Figure 21):

F55(28)Y, V100(73)I, F114(87)Y, A243(220)L, S265(242)P, N294(282)D.

[0094] The numbers in parentheses confer to the numbering of Figure 13.

[0095] In a second round, four of the seven stabilizing amino acid exchanges (E59A, R329H, S364T, G404A) found in the consensus phytase-10 sequence and, tested as single mutation in consensus phytase-1 (Table 5), were additionally introduced into the *A. fumigatus* a-mutant. Furthermore, the amino acid replacement S126N, shown to reduce the protease susceptibility of the phytase, was introduced.

[0096] The mutations were introduced as described in example 5 (see Table 6) and expressed as described in example 8 to 10. The resulting *A. fumigatus* 13073 phytase variants were called a-mutant and α-mutant-E59A-S126N-R329H-S364T-G404A.

[0097] The temperature optimum (60 °C, Figure 32) and the melting point (67.0 °C, Figure 31) of the *A. fumigatus* 13073 phytase α-mutant was increased by 5 °C in comparison to the values of the wild-type (temperature optimum: 55 °C, T_m: 60 °C). The five additional amino acid replacements further increased the temperature optimum by 3 °C (Figure 32).

Table 6: Mutagenesis primers for stabilization of *A. fumigatus* phytase ATCC 13073

Mutation	Primer
F55Y	5'-CACGTACTCGCCA**TA**CTTTTCGCTCGAG-3'
	5'-CTCGAGCGAAAAGTA**TG**GCGAGTACGTG-3'
	(*Xho* I)
E58A	5'-CCATACTTTTCG*CTCGCG*GACGAGCTGTCCGTG-3'
	5'-CACGGACAGCTCGTCC*GCGAG*CGAAAAGTAGG-3'
V100I	5'-GTATAAGAAGCTTA**TT**ACGGCGATCCAGGCC-3'
	5'-GGCCTGGATCGCCGTAA**TA**AGCTTCTTATAC-3'
F114Y	5'-CTTCAAGGGCAAGTACGCCTTTTTGAAGACG-3'
	5'-CGTCTTCAAAAAGGC**GT**ACTTGCCCTTGAAG-3'
A243L	5'-CATCCGAGCTCGC**CT**CGAGAAGCATCTTC-3'
	5'-GAAGATGCTTCTC**GA**GGCGAGCTCGGATG-3'
S265P	5'-CTAATGGATGTGT**CC**G**T**TTGATACGGTAG-3'
	5'-CTACCGTATCAAA**CG**GACACATGTCCATTAG-3'
N294D	5'-GTGGAAGAAGTA**CG**A**C**TACCTTCAGTC-3'
	5'-GACTGAAGGTAG**TC**G**T**ACTTCTTCCAC-3'
	(*Mlu* I)
R329H	5'-GCCCGGTTG*ACGCAT*TCGCCAGTGCAGG-3'
	5'-CCTGCACTGGCGA*ATGCGT*CAACCGGGC-3'

Nco I

S364T 5'-CACACGACAACA*CCATGG*TTTCCATCTTC-3'

5'-GAAGATGGAAA<u>CCATGG</u>TGTTGTCGTGTG-3'

(*Bss* HI)

G404A 5'-GTGGTGCCTTTCG*CCGCGC*GAGCCTACTTC-3'

5'-GAAGTAGGCTCG*CGCGG*CGAAAGGCACCAC-3'

Example 7

Introduction of the active site amino acid residues of the *A. niger* NRRL 3135 phytase into the consensus phytase-1

[0098] We used the crystal structure of the *Aspergillus niger* NRRL 3135 phytase to define all active site amino acid residues (see Reference Example and EP 897 010). Using the alignment of Figure 13, we replaced the following active site residues and additionally the not identical adjacent ones of the consensus phytase by that of the *A. niger* phytase:

S89D, S92G, A94K, D164S, P201S, G203A, G205S, H212P, G224A, D226T, E255T, D256E, V258T, P265S, Q292H, G300K, Y305H, A314T, S364G, M365I, A397S, S398A, G404A, and A405S

[0099] The new protein sequence consensus phytase -7 was backtranslated into a DNA sequence (Figure 22) as described in Example 3. The corresponding gene (*fcp7*) was generated as described in Example 3 using the following oligonucleotide mixes:

Mix 1.7: CP-1, CP-2, CP-3, CP-4.7, CP-5.7, CP-6, CP-7, CP-8.7, CP-9, CP-10.7

Mix 2.7: CP-9, CP-10.7, CP-11.7, CP-12.7, CP-13.7, CP-14.7, CP-15.7, CP-16, CP-17.7, CP-18.7, CP-19.7, CP-20, CR-21, CP-22.

[0100] The DNA sequences of the oligonucleotides are indicated in Figure 15 The newly synthesized oligonucleotides are additionally marked by number 7. After assembling of the oligonucleotides using the same PCR primers as mentioned in Example 3, the gene was cloned into an expression vector as described in Examples 8 - 10.

[0101] The pH-profile determined after expression in *H. polymorpha* and purification was shifted into the acidic range of the pH-spectrum showing an optimum at pH 4.5-5.0 (see Figure 30). The enzyme had a broad pH-optimum reaching at least 60% of its maximum activity from pH 2.5 to pH 6.0. Up to pH 5.0, the profile resembled the profile of the *A. niger* NRRL 3135 phytase. However, below pH 5.0 it lacked the typical low at pH 4.0 of the profile of *A. niger* phytase.

Example 8

Expression of the consensus phytase genes in *Hansenula polymorpha*

[0102] The phytase expression vectors, used to transform *H. polymorpha* RB11 (Gellissen *et al.*, 1994), was constructed by inserting the *Eco* RI fragment of pBsk⁻*fcp* or variants thereof into the multiple cloning site of the *H. polymorpha* expression vector pFPMT121, which is based on an *ura3* selection marker from *S. cerevisiae*, a formate dehydrogenase (*FMD*) promoter element and a methanol oxidase (*MO*) termimator element from *H. polymorpha*. The 5' end of the *fcp* gene is fused to the *FMD* promoter, the 3' end to the *MOX* terminator (Gellissen *et al.*, 1996; EP 0299 108 B). The resulting expression vector are designated pFPMT*fcp*, pFPMT*fcp*10, pFPMT*fcp*7.

[0103] The constructed plasmids were propagated in *E. coli*. Plasmid DNA was purified using standard state of the art procedures. The expression plasmids were transformed into the *H. polymorpha* strain RP11 deficient in orotidine-5'-phosphate decarboxylase (*ura3*) using the procedure for preparation of competent cells and for transformation of

yeast as described in Gelissen *et al.* (1996). Each transformation mixture was plated on YNB (0.14% w/v Difco YNB and 0.5% ammonium sulfate) containing 2% glucose and 1.8% agar and incubated at 37 °C. After 4 to 5 days individual transformant colonies were picked and grown in the liquid medium described above for 2 days at 37 °C. Subsequently, an aliquot of this culture was used to inoculate fresh vials with YNB-medium containing 2% glucose. After seven further passages in selective medium, the expression vector integrates into the yeast genome in multimeric form. Subsequently, mitotically stable transformants were obtained by two additional cultivation steps in 3 ml non-selective liquid medium (YPD, 2% glucose, 10 g yeast extract, and 20 g peptone). In order to obtain genetically homogeneous recombinant strains an aliquot from the last stabilization culture was plated on a selective plate. Single colonies were isolated for analysis of phytase expression in YNB containing 2% glycerol instead of glucose to derepress the *fmd* promoter. Purification of the consensus phytases was done as described in Example 9.

Example 9

Expression of the consensus phytase genes in *Saccharomyces cerevisiae* and purification of the phytases from culture supernatant

[0104] The consensus phytase genes were isolated from the corresponding Bluescript-plasmid (pBsk⁻*fcp*, pBSK⁻*fcp10*, pBsk⁻*fcp7*) and ligated into the *Eco* RI sites of the expression cassette of the *Saccharomyces cerevisiae* expression vector pYES2 (Invitrogen, San Diego, CA, USA) or subcloned between the shortened GAPFL (glyceraldhyde-3-phosphate dehydrogenase) promoter and the *pho5* terminator as described by Janes *et al.* (1990). The correct orientation of the gene was checked by PCR. Transformation of *S. cerevisiae* strains. e. g. INVSc1 (Invitrogen, San Diego, CA, USA) was done according to Hinnen *et al.* (1978). Single colonies harboring the phytase gene under the control of the GAPFL promoter were picked and cultivated in 5 ml selection medium (SD-uracil, Sherman *et al.*, 1986) at 30°C under vigorous shaking (250 rpm) for one day. The preculture was then added to 500 ml YPD medium (Sherman *et al.*, 1986) and grown under the same conditions. Induction of the *gal1* promoter was done according to manufacturer's instruction. After four days of incubation cell broth was centrifuged (7000 rpm, GS3 rotor, 15 mm, 5°C) to remove the cells and the supernatant was concentrated by way of ultrafiltration in Amicon 8400 cells (PM30 membranes) and ultrafree-15 centrifugal filter devices (Biomax-30K, Millipore, Bedford, MA, USA). The concentrate (10 ml) was desalted on a 40 ml Sephadex G25 Superfine column (Pharmacia Biotech, Freiburg, Germany), with 10 mM sodium acetate, pH 5.0, serving as elution buffer. The desalted sample was brought to 2 M $(NH_4)_2SO_4$ and directly loaded onto a 1 ml Butyl Sepharose 4 Fast Flow hydrophobic interaction chromatography column (Pharmacia Biotech, Feiburg, Germany) which was eluted with a linear gradient from 2 M to 0 M $(NH4)_2SO_4$ in 10 mM sodium acetate, pH 5.0. Phytase was eluted in the break-through, concentrated and loaded on a 120 ml Sephacryl S-300 gel permeation chromatography column (Pharmacia Biotech, Freiburg, Germany). Consensus phytase and consensus phytase -7 eluted as a homogeneous symmetrical peak and was shown by SDS-PAGE to be approx. 95% pure.

Example 10

Expression of the consensus phytase genes in *Aspergillus niger*

[0105] The Bluescript-plasmids pBsk⁻*fcp*, pBSK⁻*fcp10*, and pBsk⁻*fcp7* were used as template for the introduction of a *Bsp* HI-site upstream of the start codon of the genes and an *Eco* RV-site downstream of the stop codon. The Expand™ High Fidelity PCR Kit (Boehringer Mannheim, Mannheim, Germany) was used with the following primers:

Primer Asp-1:

Bsp HI

5'-TATA*TCATGA*GCGTGTTCGTCGTGCTACTGTTC-3'

Primer Asp-2 used for cloning of *fcp* and *fcp7*:

Eco RV

3'-ACCCGACTTACAAAGCGAATT*CTATAG*ATATAT-5'

Primer Asp-3 used for cloning of *fcp10*:

Eco RV

3'-ACCCTTCTTACAAAGCGAATT*CTATAG*ATATAT-5'

[0106] The reaction was performed as described by the supplier. The PCR-amplified *fcp*-genes had a new *Bsp* HI site at the start codon, introduced by primer Asp-1, which resulted in a replacement of the second amino acid residue glycine by seine. Subsequently, the DNA-fragment was digested with *Bsp* HI and *Eco* RV and ligated into the *Nco* I site downstream of the glucoamylase promoter of *Aspergillus niger* (*glaA*) and the *Eco* RV site upstream of the *Aspergillus nidulans* tryptophan C terminator (*trpC*) (Mullaney *et al.*, 1985). After this cloning step, the genes were sequenced to detect possible failures introduced by PCR. The resulting expression plasmids which basically corresponds to the pGLAC vector as described in Example 9 of EP 684 313, contained the orotidine-5'-phosphate decarboxylase gene (*pyr4*) of *Neurospora crassa* as a selection marker. Transformation of *Aspergillus niger* and expression of the consensus phytase genes was done as described in EP 684 313. The consensus phytases were purified as described in Example 9.

Example 11

Determination of phytase activity and of temperature optimum

[0107] Phytase activity was determined basically as described by Mitchell et al (1997). The activity was measured in an assay mixture containing 0.5% phytic acid (≈5 mM) in 200 mM sodium acetate, pH 5.0. After 15 mm of incubation at 37 °C, the reaction was stopped by addition of an equal volume of 15% trichloroacetic acid. The liberated phosphate was quantified by mixing 100 µl of the assay mixture with 900 µl H_2O and 1 ml of 0.6 M H_2SO_4, 2% ascorbic acid and 0.5% ammonium molybdate. Standard solutions of potassium phosphate were used as reference. One unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol phosphate per minute at 37 °C. The protein concentration was determined using the enzyme extinction coefficient at 280 nm calculated according to Pace et al (1995): consensus phytase, 1.101; consensus phytase 7, 1.068; consensus phytase 10, 1.039.

[0108] In case of pH-optimum curves, purified enzymes were diluted in 10 mM sodium acetate, pH 5.0. Incubations were started by mixing aliquots of the diluted protein with an equal volume of 1% phytic acid (≈10 mM) in a series of different buffers: 0.4 M glycine/HCl, pH 2.5; 0.4 M acetate/NaOH, pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5; 0.4 M imidazole/HCl, pH 6.0, 6.5; 0.4 M Tris/HCl pH 7.0, 7.5, 8.0, 8.5, 9.0. Control experiments showed that pH was only slightly affected by the mixing step. Incubations were performed for 15 min at 37 °C as described above.

[0109] For determinations of the substrate specificities of the phytases, phytic acid in the assay mixture was replaced by 5 mM concentrations of the respective phosphate compounds. The activity tests were performed as described above.

[0110] For determination of the temperature optimum, enzyme (100 µl) and substrate solution (100 µl) were pre-incubated for 5 mm at the given temperature. The reaction was started by addition of the substrate solution to the enzyme. After 15 min incubation, the reaction was stopped with trichloroacetic acid and the amount of phosphate released was

determined.

[0111] The pH-optimum of the original consensus phytase was around pH 6.0-6.5 (70 U/mg). By introduction of the Q50T mutation, the pH-optimum shifted to pH 6.0 (130 U/mg). After introduction of K91A, the pH optimum shifted one pH-unit into the acidic pH-range showing a higher specific activity between pH 2.5 and pH 6.0. That was shown for the stabilized mutants and for consensus phytase-10, too (Figure 26 and 27).

[0112] Consensus phytase-7, which was constructed to transfer the catalytic characteristics of the *A. niger* phytase NRRL 3135 into the consensus phytase, had a pH-profile which is shifted into the acidic range of the pH-spectrum showing an optimum between pH 4.5 and 5.0 (see Figure 31). The enzyme had a broad pH-optimum reaching at least 60% of its increased maximum activity from pH 2.5 to pH 6.0. The substrate spectrum, too, resemble more to that of the A. niger NRRL 3135 phytase than to the consensus phytase-1.

[0113] The temperature optimum of consensus phytase-1 (71 °C) was 16-26 °C higher than the temperature optimum of the wild-type phytases (45-55 °C, Table 7) which were used to calculate the consensus sequence. The improved consensus phytase-10 showed a further increase of its temperature optimum to 80 °C (Figure 33). The temperature optimum of the consensus phytase-1-thermo[8] was found in the same range (78 °C) using the supernatant of an overproducing *S. cerevisiae* strain. The highest temperature optimum reached of 82 °C was determined for consensus phytase-10-thermo-Q50T-K91A.

Table 7

Temperature optimum and T_m-value of consensus phytase and of the phytases from *A. fumigatus*, *A. niger*, *E. nidulans*, and *M. thermophila*. The determination of the temperature optimum was performed as described in Example 11 The T_m-values were determined by differential scanning calorimetry as described in Example 12.

phytase	temperature optimum [°C]	*T*m [°C]
Consensus phytase-10-thermo-Q50T-K91A	82	89.3
Consensus phytase-10-thermo-Q50T	82	88.6
Consensus phytase-10	80	85.4
Consensus phytase-1-thermo[8]-Q50T	78	84.7
Consensus phytase-1-thermo[8]-Q50T-K91A	78	85.7
Consensus phytase-1	71	78.1
A. niger NRRL3135	55	63.3
A. fumigatus 13073	55	62.5
A. fumigatus 13073 α-mutant	60	67.0
A. fumigatus 13073 α-mutant (optimized)	63	-
A. terreus 9A-1	49	57.5
A. terreus cbs.116.46	45	58.5
E. nidulans	45	55.7
M. thermophila	55	n. d.
T. thermophilus	45	n. d.

Example 12

Determination of the melting point by differential scanning calorimetry (DSC)

[0114] In order to determine the unfolding temperature of the phytases, differential scanning calorimetry was applied as previously published by Brugger et al (1997). Solutions of 50-60 mg/ml homogeneous phytase were used for the

tests. A constant heating rate of 10 °C/min was applied up to 90-95 °C.

[0115] The determined melting points reflect the results obtained for the temperature optimums (Table 7). The most stable consensus phytase designed is consensus phytase-10-thermo-Q50T-K91A showing a melting temperature under the choosen condition of 89.3 C. This is 26 to 33.6 °C higher than the melting point of the wild-type phytases used.

Example 13

Transfer of basidiomycete phytase active site into consensus phytase-10-thermo-Q50T-K91A

[0116] As described previously (Example 5), mutations derived from the basidiomycete phytase active site were introduced into the consensus phytase 10. The following five constructs a) to e) were prepared:

[0117] This construct is called consensus phytase 12, and it comprises a selected number of active site residues of the basidio consensus sequence, its amino acid sequence (consphy12) is shown in Fig. 33 (the first 26 amino acids forms the signal peptide, amended positions are underlined);

a cluster of mutations (Cluster II) was transferred to the consensus 10 sequence, viz.: S80Q, Y86F, S90G, K91A, S92A, K93T, A94R, Y95I;

analogously, another cluster of mutations (Cluster III) was transferred, viz.: T129V, E133A, Q143N, M136S, V137S, N138Q, S139A;

analogously, a further cluster of mutations (Cluster IV) was transferred, viz.: A168D, E171T, K172N, F173W;

and finally, a further cluster of mutations (Cluster V) was transferred, viz.: Q297G, S298D, G300D, Y305T.

[0118] These constructs were expressed as described in Examples 8 to 10.

References:

[0119]

Akanuma, S., Yamagishi, A., Tanaka, N. & Oshima, T. (1998). Serial increase in the thermal stability of 3-isopropylmalate dehydrogenase from *Bacillus subtilis* by experimental evolution. *Prot. Sci.* **7**, 698-705.

Arase, A., Yomo, T., Urabe, I., Hata, Y., Katsube, Y. & Okada, H. (1993). Stabilization of xylanase by random mutagenesis. *FEBS Lett.* **316**, 123-127.

Berka, R. M., Rey, M. W., Brown, K. M., Byun, T. & Klotz, A. V. (1998). Molecular characterization and expression of a phytase gene from the thermophilic fungus Thermomyces lanuginosus. *Appl. Environ. Microbiol.* **64**, 4423-4427.

Blaber, M., Lindstrom, J. D., Gassner, N., Xu, J., Heinz, D. W. & Matthews, B. W. (1993). Energetic cost and structural consequences of burying a hydroxyl group within the core of a protein determined from Ala'Ser and Val'Thr substitutions in T4 lysozyme. *Biochemistry* **32**, 11363-11373.

Brugger, R., Mascarello, F., Augem, S., van Loon, A. P. G. M. & Wyss, M. (1997). Thermal denaturation of phytases and pH 2.5 acid phosphatase studied by differential scanning calorimetry. In *The Biochemistry of phytate and phytase* (eds. *Rasmussen, S.K*; *Raboy, V.*; *Dalbøge, H.* and *Loewus, F.*; Kluwer Academic Publishers.

Cosgrove, D.J. (1980) Inositol phosphates - their chemistry, biochemistry and physiology: studies in organic chemistry, chapter 4. Elsevier Scientific Publishing Company, Amsterdam, Oxford, New York.

Devereux, J., Haeberli, P.& Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**, 387-395.

Gellissen, G., Hollenberg, C. P., Janowicz, Z. A. (1994) Gene expression in methylotrophic yeasts . In: Smith, A. (ed.) Gene expression in recombinant microorganisms. Dekker, New York, 395-439.

Gellissen, G., Piontek, M., Dahlems, U., Jenzelewski, V., Gavagan, J. E., DiCosimo, R., Anton, D. I. & Janowicz, Z. A. (1996) Recombinant *Hansenula polymorpha* as a biocatalyst: coexpression of the spinach glycolate oxidase (*GO*) and the *S. cerevisiae* catalase T (*CTT1*) gene. *Appl. Microbiol. Biotechnol.* **46**, 46-54.

Gerber, P. and Müller, K. (1995) Moloc molecular modeling software. *J. Comput. Aided Mol. Des.* **9**, 251-268

Hinnen, A., Hicks, J. B. & Fink, G, R. (1978) Transformation of yeast. *Proc. Natl. Acad. Sci.* USA **75**, 1929-1933.

Imanaka, T., Shibazaki, M. & Takagi, M. (1986). A new way of enhancing the thermostability of proteases. *Nature* **324**, 695-697.

Janes, M., Meyhack, B., Zimmermann, W. & Hinnen, A. (1990) The influence of GAP promoter variants on hirudine production, average plasmid copy number and cell growth in *Saccharomyces cerevisiae. Curr Genet.* **18**, 97-103.

Karpusas, M., Baase, W. A., Matsumura, M. & Matthews, B. W. (1989). Hydrophobic packing in T4 lysozyme probed by cavity-filling mutants. *Proc. Natl. Acad Sci.(USA)* **86**, 8237-8241.

Margarit, I., Campagnoli, S., Frigerio, F., Grandi, G., Fillipis, V. D. & Fontana, A. (1992). Cumulative stabilizing effects of glycine to alanine substitutions in *Bacillus subtilis* neutral protease. *Prot. Eng.* **5**, 543-550.

Matthews, B. W. (1987a). Genetic and structural analysis of the protein stability problem. *Biochemistry* **26**, 6885-6888.

Matthews, B. W. (1993). Structural and genetic analysis of protein stability. *Annu. Rev. Biochem.* **62**, 139-160.

Matthews, B. W., Nicholson, H. & Becktel, W. (1987). Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding. *Proc. Natl. Acad. Sci. (USA)* **84**, 6663-6667.

Mitchell, D. B., Vogel, K., Weimann, B. I., Pasamontes, L. & van Loon, A. P. G. M. (1997) The phytase subfamily of histidine acid phosphatases: isolation of genes for two novel phytases from the fungi *Aspergillus terreus* and *Myceliophthora thermophila*, *Microbiology* **143**, 245-252.

Mullaney, E. J., Hamer, J. E., Roberti, K. A., Yelton, M. M. & Timberlake, W. E. (1985) Primary structure of the *trpC* gene from *Aspergillus nidulans*. *Mol. Gen. Genet.* **199**, 37-46.

Munoz, V. & Serrano, L. (1995). Helix design, prediction and stability. *Curr. Opin. Biotechnol.* **6**, 382-386.

Pace, N. C., Vajdos, F., Fee, L., Grimsley, C. & Gray, T. (1995). How to measure and predict the molar absorption coefficient of a protein. *Prot. Sci.* **4**, 2411-2423.

Pantoliano, M. W., Landner, R. C., Brian, P. N., Rollence, M. L., Wood, J. F. & Poulos, T. L. (1987). Protein engineering of subtilisin BPN': enhanced stabilization through the introduction of two cysteines to form a disulfide bond. *Biochemistry* **26**, 2077-2082.

Pasamontes, L., Haiker, M., Henriquez-Huecas, M., Mitchell, D. B. & van Loon, A. P. G. M. (1997a). Cloning of the phytases from *Emericella nidulans* and the thermophilic fungus *Talaromyces thermophilus*. *Biochim. Biophys. Acta* **1353**, 217-223.

Pasamontes, L., Haiker, M., Wyss, M., Tessier, M. & van Loon, A. P. G. M. (1997) Cloning, purification and characterization of a heat stable phytase from the fungus *Aspergillus fumigatus*, *Appl. Environ. Microbiol.* **63**, 1696-1700.

Piddington, C. S., Houston, C. S., Paloheimo, M., Cantrell, M., Miettinen-Oinonen, A. Nevalainen, H., & Rambosek, J. (1993) The cloning and sequencing of the genes encoding phytase (*phy*) and pH 2.5-optimum acid phosphatase (*aph*) from *Aspergillus niger* var. *awamori*. *Gene* **133**, 55-62.

Purvis, I. J., Bettany, A. J. E., Santiago, T. C., Coggins, J. R., Duncan, K., Eason, R. & Brown, A. J. P. (1987). The efficiency of folding of some proteins is increased by controlled rates of translation in vivo. *J. Mol. Biol.* **193**, 413-417.

Risse, B., Stempfer, G., Rudolph, R., Schumacher, G. & Jaenicke, R. (1992). Characterization of the stability effect of point mutations of pyruvate oxidase from *Lactobacillus plantarum*: protection of the native state by modulating coenzyme binding and subunit interaction. *Prot. Sci.* **1**, 1710-1718.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sauer, R., Hehir, K., Stearman, R., Weiss, M., Jeitler-Nilsson, A., Suchanek, E. & Pabo, C. (1986). An engineered intersubunit disulfide enhances the stability and DNA binding of the N-terminal domain of 1-repressor. *Biochemistry* **25**, 5992-5999.

Serrano, L., Day, A. G. & Fersht, A. R. (1993). Step-wise mutation of barnase to binase. A procedure for engineering increased stability of proteins and an experimental analysis of the evolution of protein stability. *J. Mol. Biol.* **233**, 305-312.

Sheman, J. P., Finck, G. R. & Hicks, J. B. (1986) Laboratory course manual for methods in yeast genetics. Cold Spring Harbor University.

Steipe, B., Schiller, B., Plueckthun, A. & Steinbach, S. (1994). Sequence statistics reliably predict stabilizing mutations in a protein domain. *J. Mol. Biol.* **240**, 188-192.

van den Burg, B., Vriend, G., Veltman, O. R., Venema & G., Eijsink, V. G. H. (1998). Engineering an enzyme to resist boiling. *Proc. Natl. Acad. Sci.* (USA) **95**, 2056-2060.

Van Etten, R.L. (1982) Human prostatic acid phosphatase: a histidine phosphatase. *Ann. NY Acad. Sci.* **390**,27-50

van Hartingsveldt, W., van Zeijl, C. M. F., Harteveld, G. M., Gouka, R. J., Suykerbuyk, M. E. G., Luiten, R. G. M., van Paridon, P. A., Selten, G. C. M., Veenstra, A. E., van Gorcom, R. F. M., & van den Hondel, C. A. M. J. J. (1993) Cloning, characterization and overexpression of the phytase-encoding gene (*phyA*) of *Aspergillus niger*. *Gene* **127**, 87-94.

van Loon, A. P. G. M., Simoes-Nunes, C., Wyss, M., Tomschy, A., Hug, D., Vogel, K. & Pasamontes, L. (1998). A heat resistant phytase of *Aspergillus fumigatus* with superior performance in animal experiments. Phytase optimization and natural variability. In *the Biochemistry of phytate and phytases*. Kluwer Academic Press, s.a.

Claims

1. A stabilized dry or liquid enzyme formulation comprising phytase and one or more stabilizing agents selected from the group consisting of:

    a) C_5 sugars, preferably xylitol or ribitol,

    b) polyethylene glycols having a molecular weight of 600 to 4000 Da, preferably 1000 to 3350 Da.

    c) the disodium salts of malonic, glutaric and succinic acid,

    d) carboxymethylcellulose, and

    e) alginate, preferably sodium alginate.

2. A stabilized dry or liquid enzyme formulation comprising phytase which has been crosslinked:

    a) with glutaraldehyde, or by
    b) oxidation with sodium periodate and reaction with adipic acid dihydrazide.

3. Enzyme formulation according to claims 1 or 2, characterized in that the phytase is a fungal or a consensus phytase.

4. Enzyme formulation according to claim 3, characterized in that the fungal phytase is selected from the group consisting of *Aspergillus fumigatus, Aspergillus nidulans*, *Aspergillus terreus* and *Aspergillus niger* phytase.

5. Enzyme formulation according to anyone of claims 1 to 4 characterized in that the formulation is liquid.

6. Enzyme formulation according to claim 5, characterized in that the stabilizing agent is polyethylene glycol whereby the polyethylene glycol is present in a concentration of 10-50% (w/w) in the final formulation.

7. Enzyme formulation according to claim 5 or 6, characterized in that the stabilizing agent is xylitol and/or ribitol which is present in the final formulation in a concentration of 20-60% (w/w).

8. Enzyme formulation according to any of claims 5 to 7, characterized in that the stabilizing agent is the disodium salt of glutaric, succinic or malonic acid whereby the concentration of the salt in the final formulation ranges between 10 and 30% (w/w).

9. Enzyme formulation according to any of claims 5 to 8, characterized in that the stabilizing agent is carboxymethylcellulose whereby the concentration of the polymer in the final formulation ranges between 1 and 10% (w/w).

10. Enzyme formulation according to any of claims 5 to 9, characterized in that the stabilizing agent is sodium alginate whereby the concentration of the polymer in the final formulation ranges between 1 and 10% (w/w).

11. Enzyme formulation according to any of claims 1-4, characterized in that the formulation is dry/solid.

12. Enzyme formulation according to claim 11, characterized in that the stabilizing agent is polyethylene glycol whereby the polyethylene glycol is present in a concentration of 1-20% (w/w) in the final formulation.

13. Enzyme formulation according to claim 11 or 12, characterized in that the stabilizing agent is xylitol and/or ribitol which is present in the final formulation in a concentration of 1-20% (w/w).

14. Enzyme formulation according to any of claims 11 to 13, characterized in that the stabilizing agent is the disodium salt of glutaric, succinic or malonic acid whereby the concentration of the salt in the final formulation ranges between 1 and 20% (w/w).

15. Enzyme formulation according to any of claims 11 to 14, characterized in that the stabilizing agent is carboxymethylcellulose whereby the concentration of the polymer in the final formulation ranges between 1 and 10% (w/w).

16. Enzyme formulation according to any of claims 11 to 15, characterized in that the stabilizing agent is sodium alginate whereby the concentration of the polymer in the final formulation ranges between 1 and 10% (w/w).

17. Enzyme formulation according to any of claims 2-5 or 11 characterized in that the phytase monomers are crosslinked by addition of glutaraldehyde.

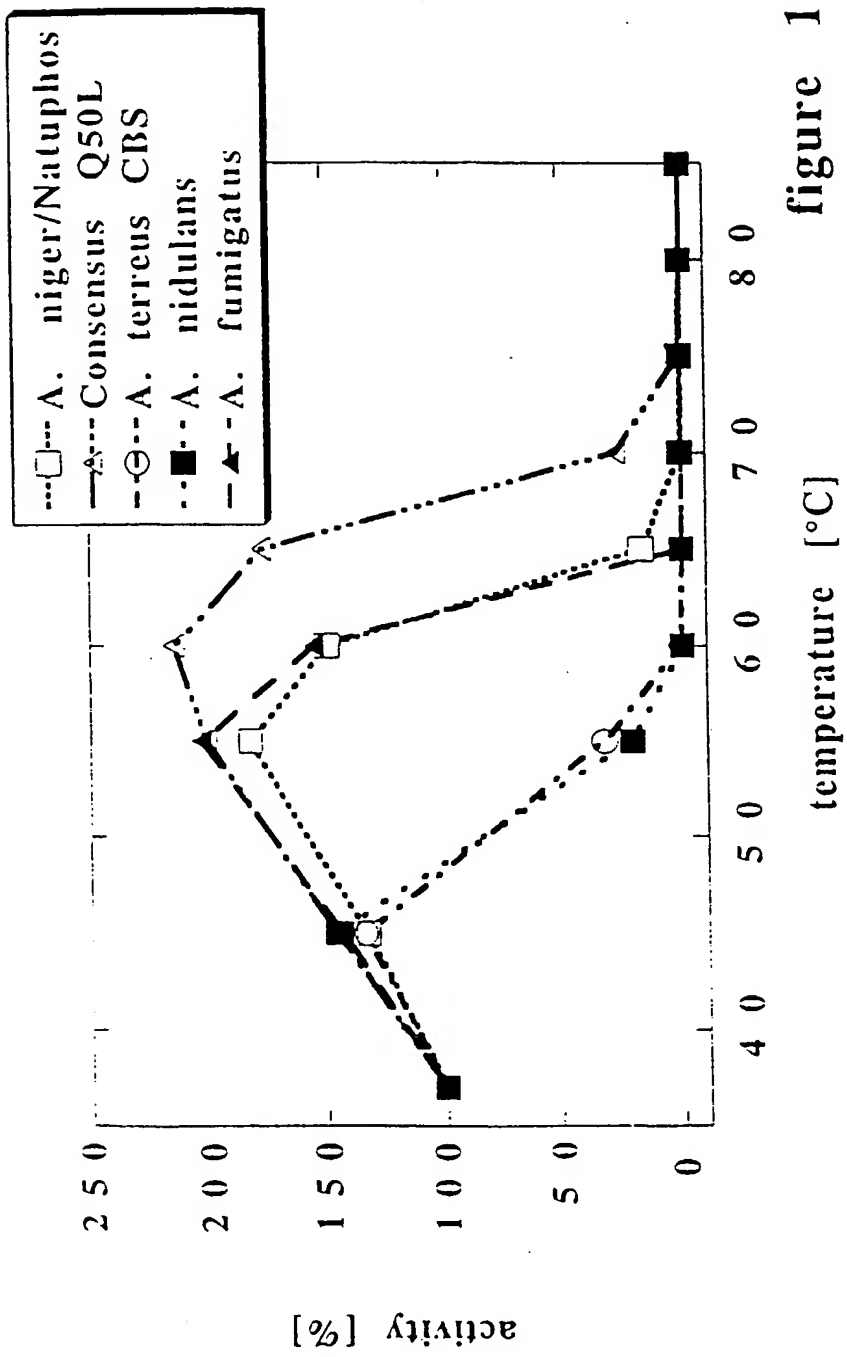
18. Enzyme formulation according to any of claims 2-5 or 11 characterized in that the phytase monomers are crosslinked by oxidation of carbohydrate residues with sodium periodate and subsequent addition of adipic acid dihydrazide.

19. A method of preparing a feed composition for monogastric animals, characterized in that the feed is treated with a stabilized dry or liquid enzyme formulation according to any of claims 1-18.

20. A feed composition for monogastric animals, characterized in that the feed comprises a stabilized dry or liquid enzyme formulation according to any one of claims 1-18.

21. A method of providing a monogastric animal with its dietary requirement of phosphorous, characterized in that the animal is feeded with a feed according to claim 20 and that no additional phosphorous is added to the feed.

22. A method of preparing a dry or liquid phytase formulation, characterized in that a stabilized phytase according to claims 1-18 is used.


A. niger/Natuphos
Consensus Q50L
A. terreus CBS
A. nidulans
A. fumigatus
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50
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40
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70
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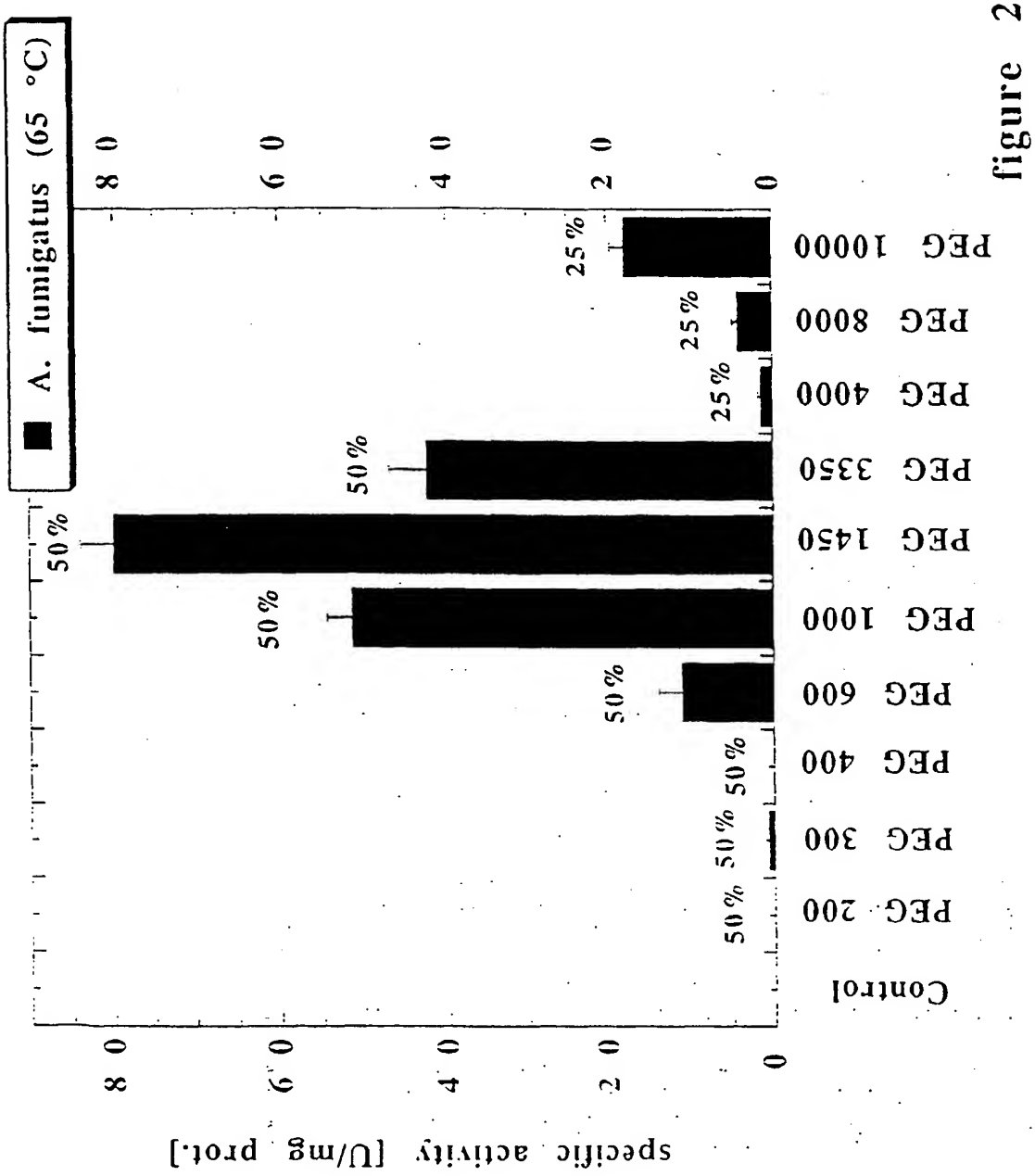
temperature [°C]

figure 1



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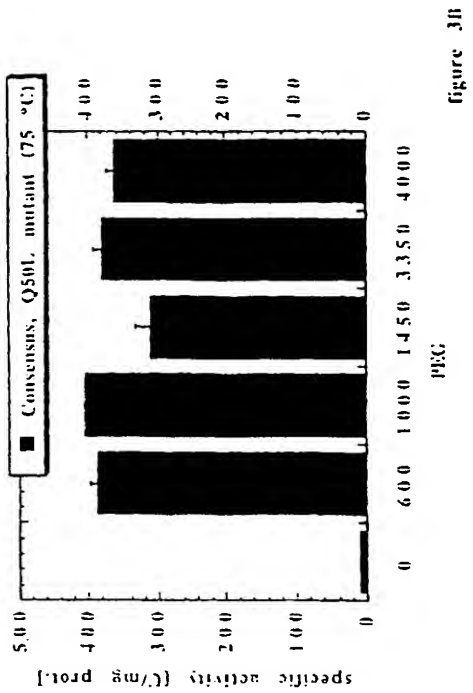

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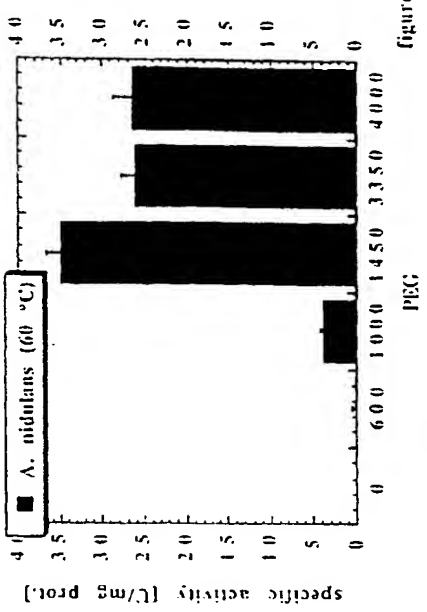
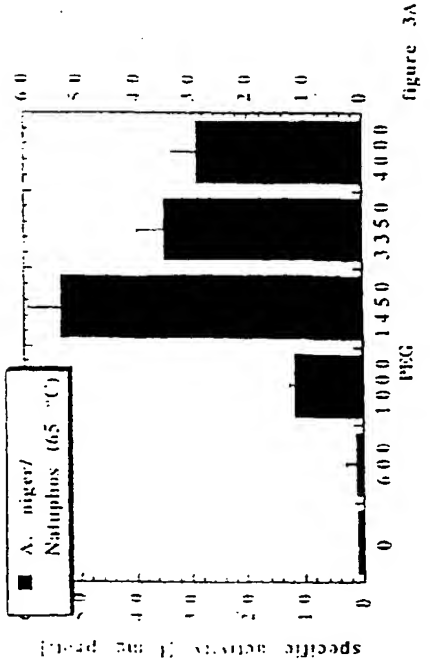


figure 3D



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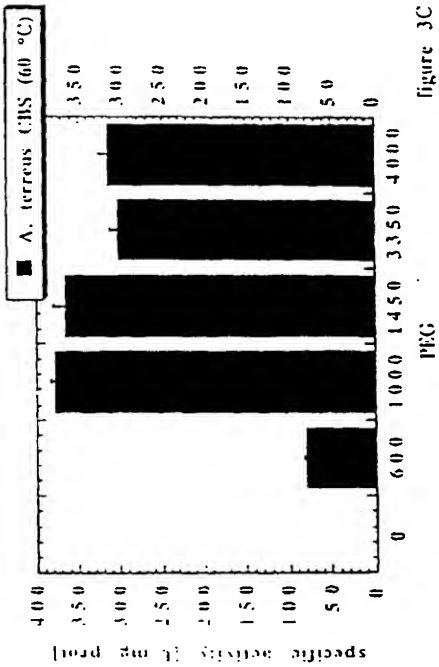


figure 3C

figure 3

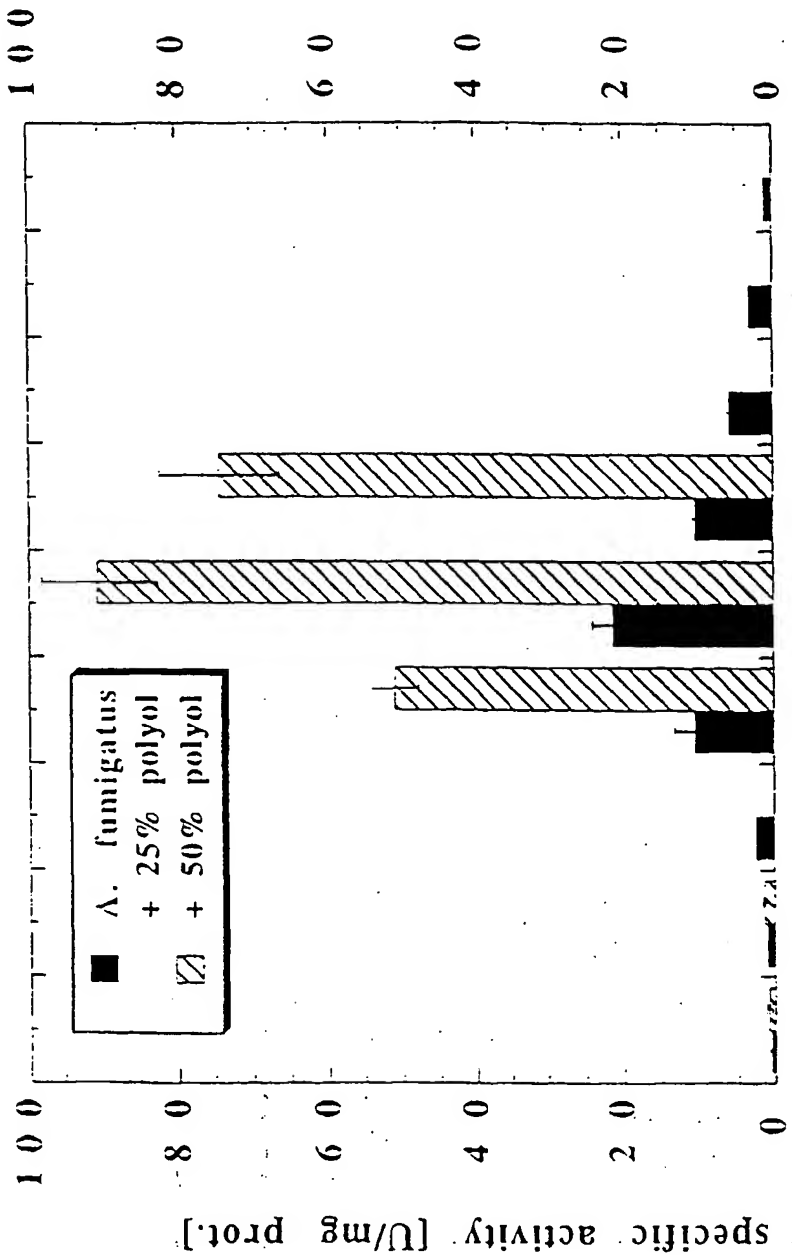


figure 4

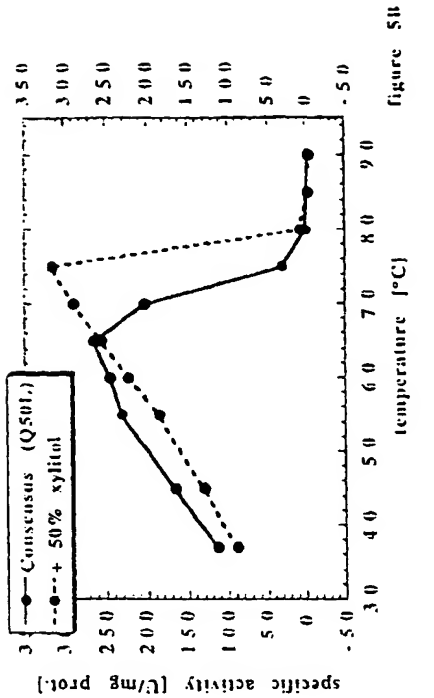
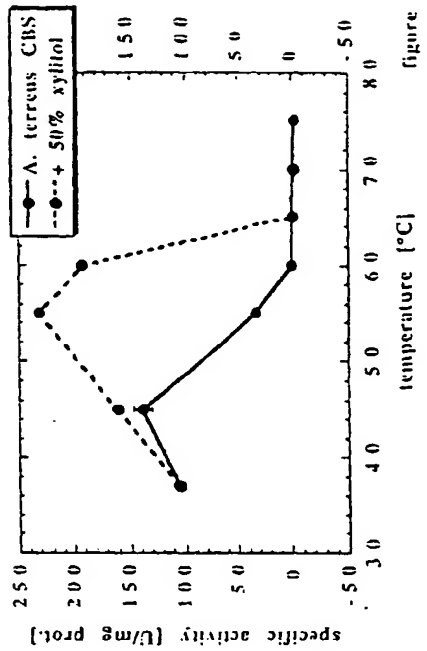


figure 5B



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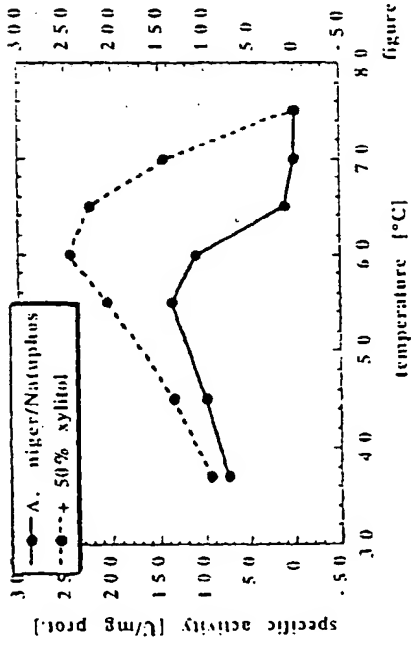

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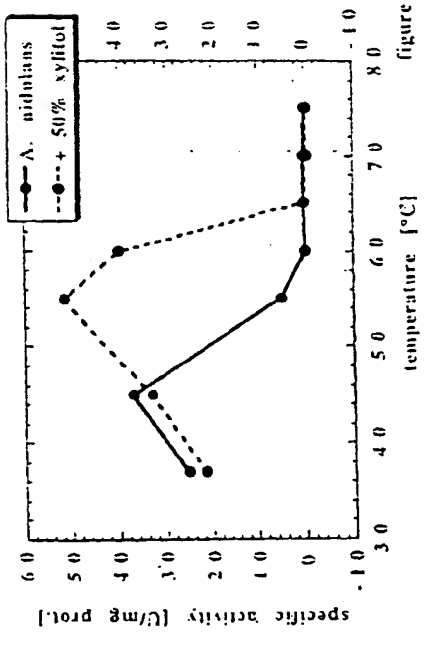
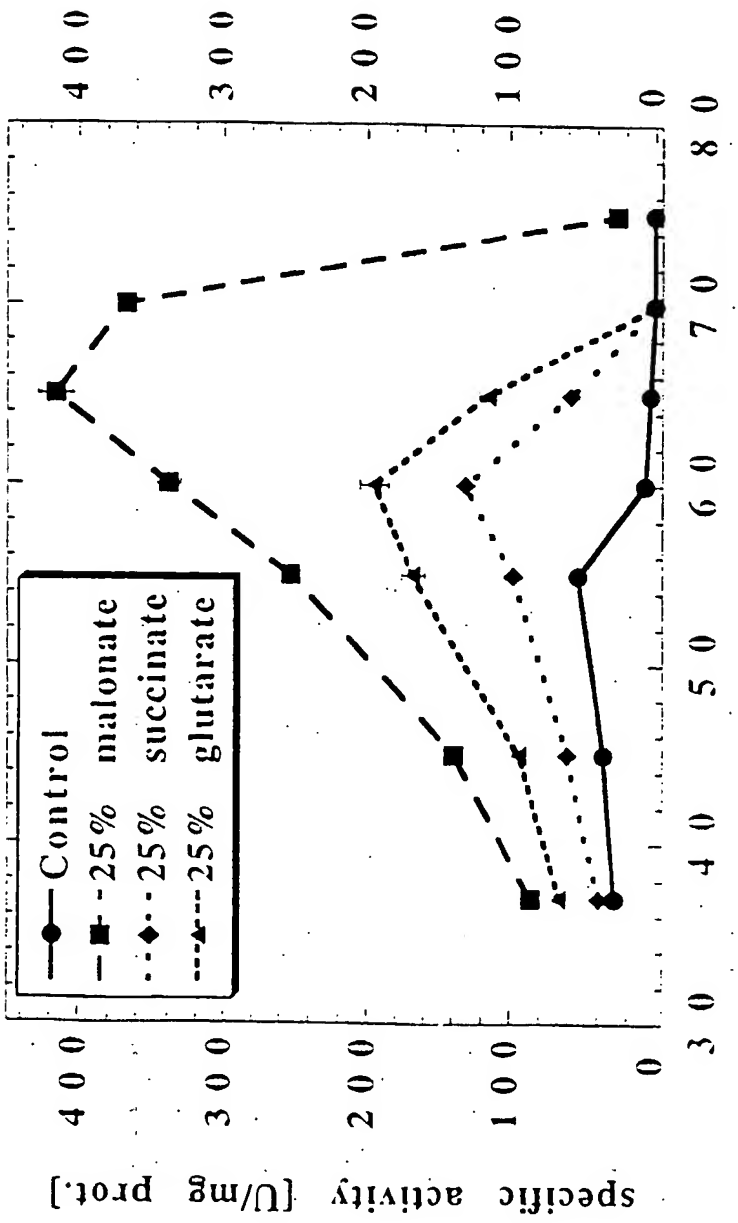


figure 5C

figure 5


specific activity [U/mg prot.]
0
100
200
300
400
30
40
50
60
70
80
temperature [°C]
Control
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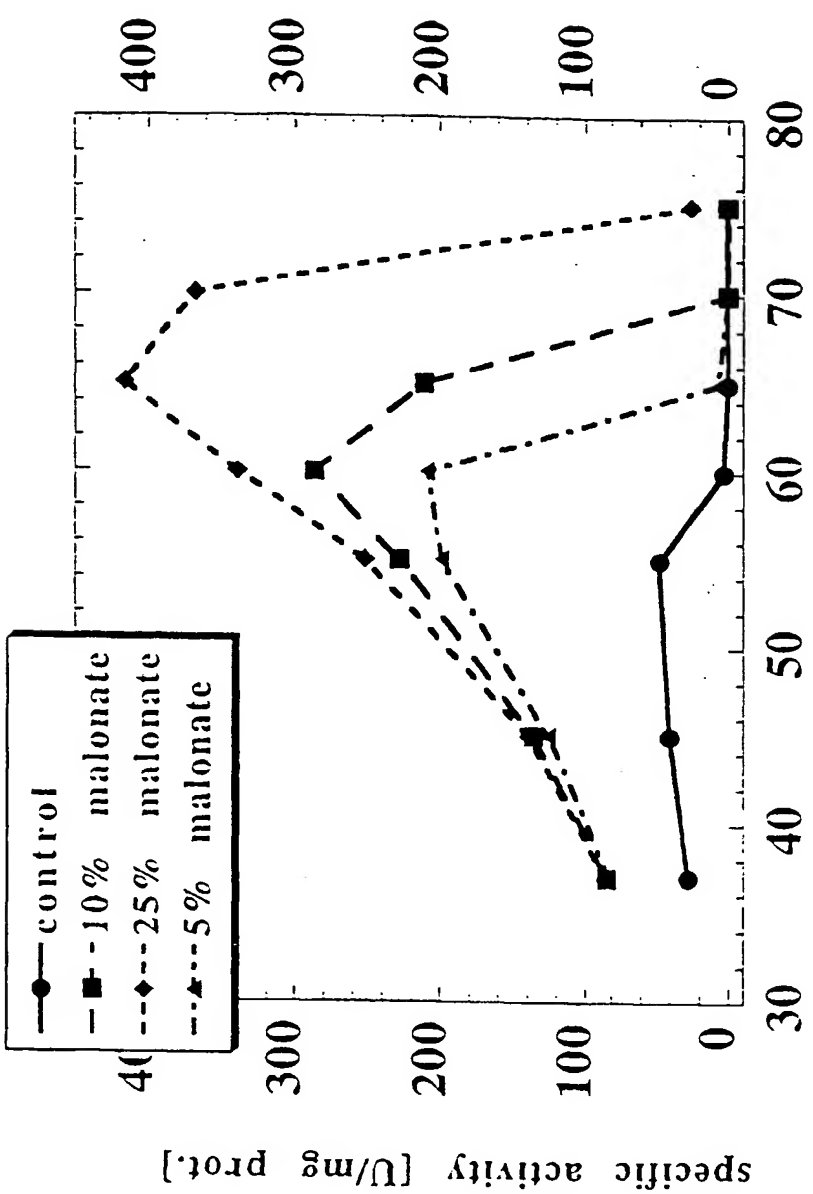
-25% succinate
-25% glutarate

figure 6


control
10% malonate
25% malonate
5% malonate
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temperature [°C]
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100
200
300
400
30
40
50
60
70
80

figure 7

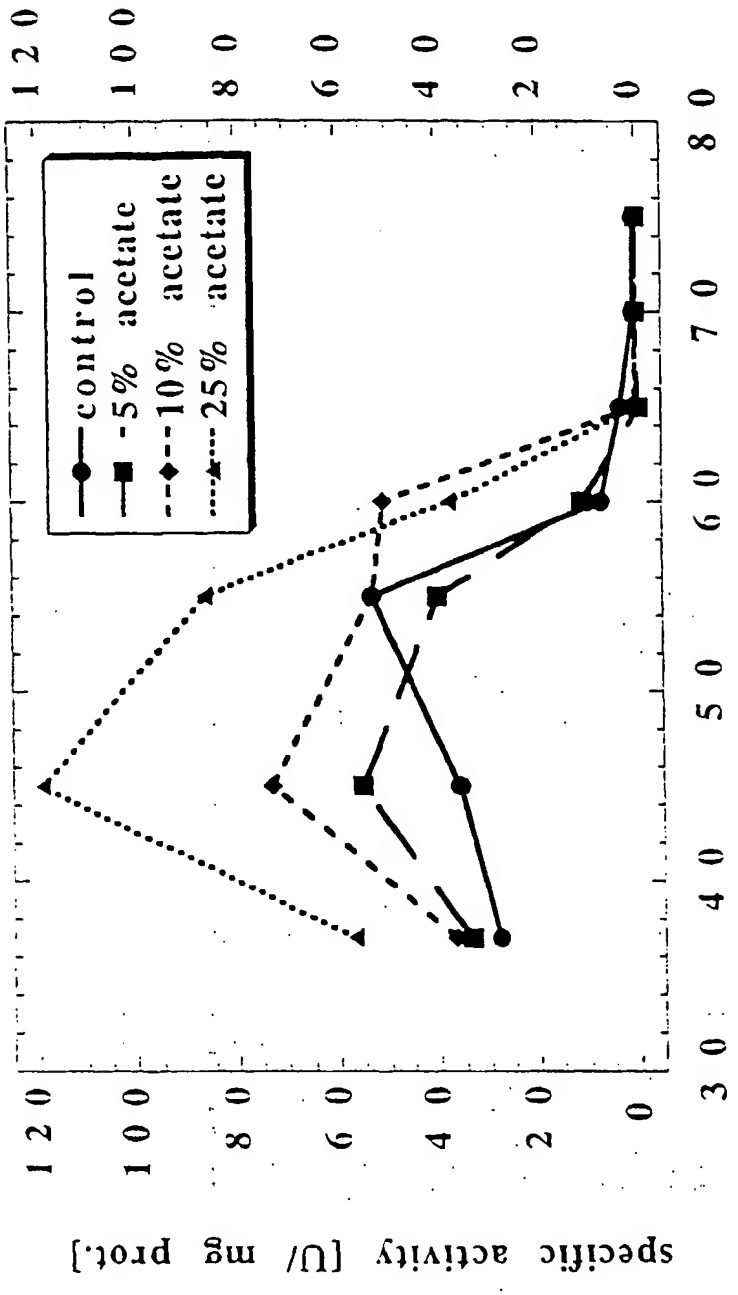

figure 8

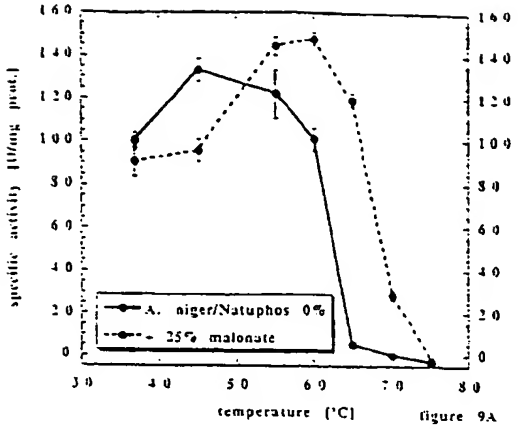

figure 9A

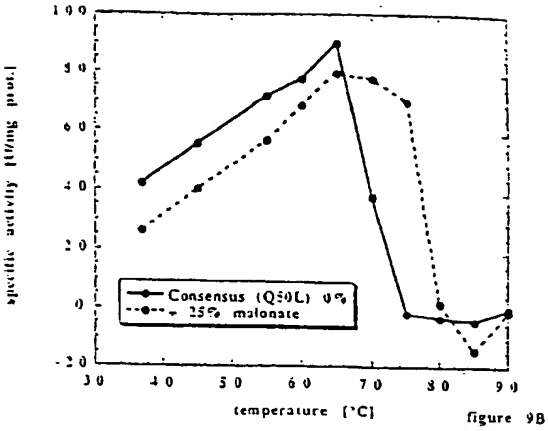

figure 9B

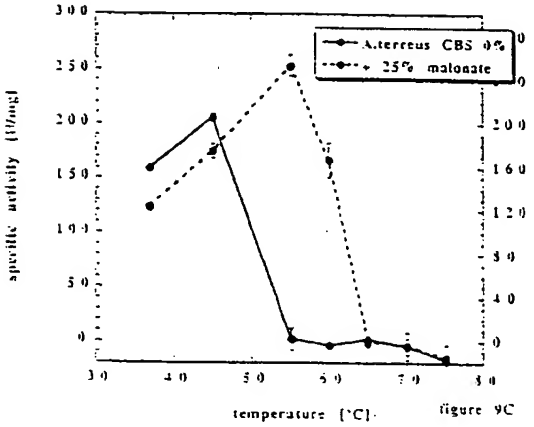

figure 9C

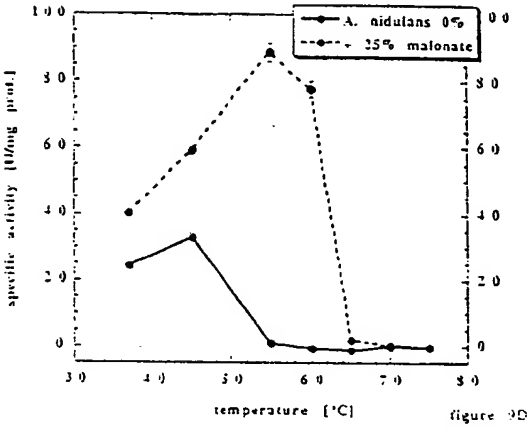

figure 9D

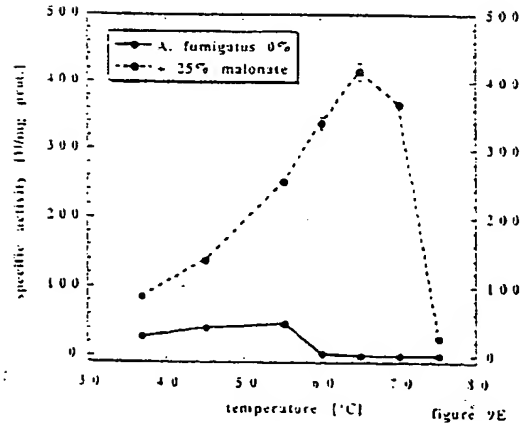

figure 9E

Figure 9

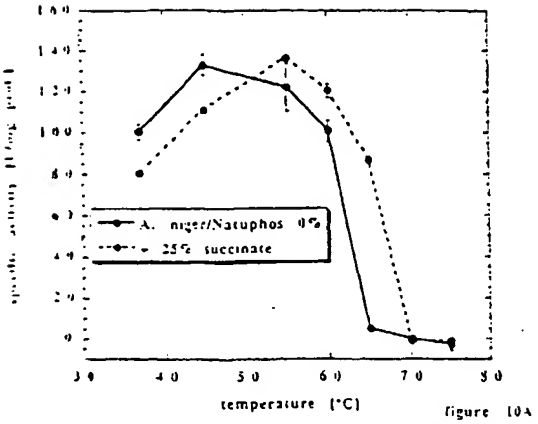

figure 10A

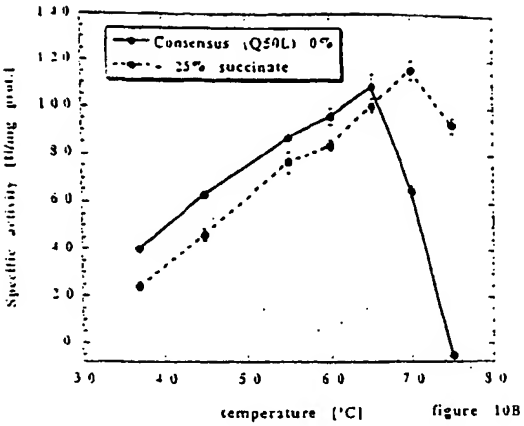

figure 10B

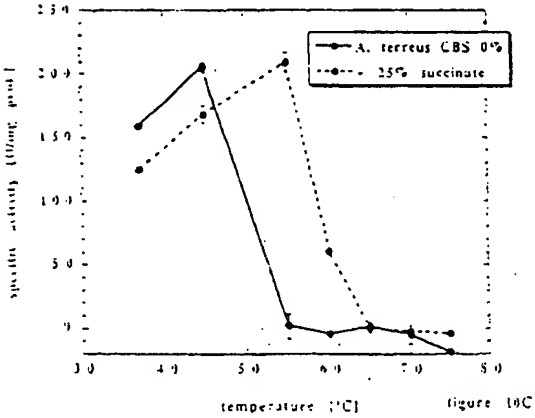

figure 10C

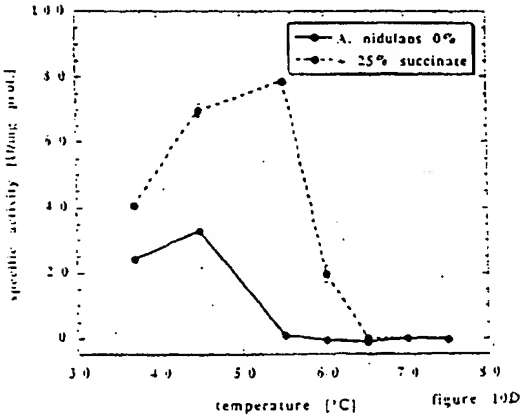

figure 10D

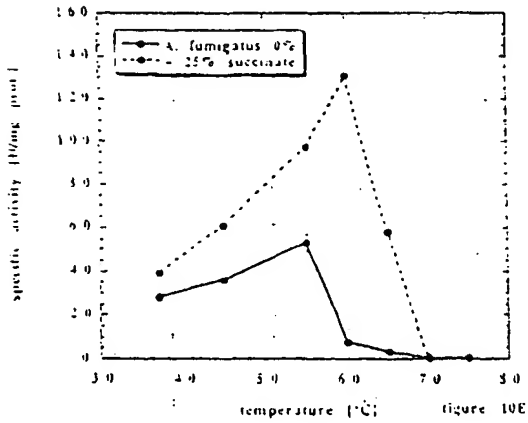

figure 10E

Figure 10

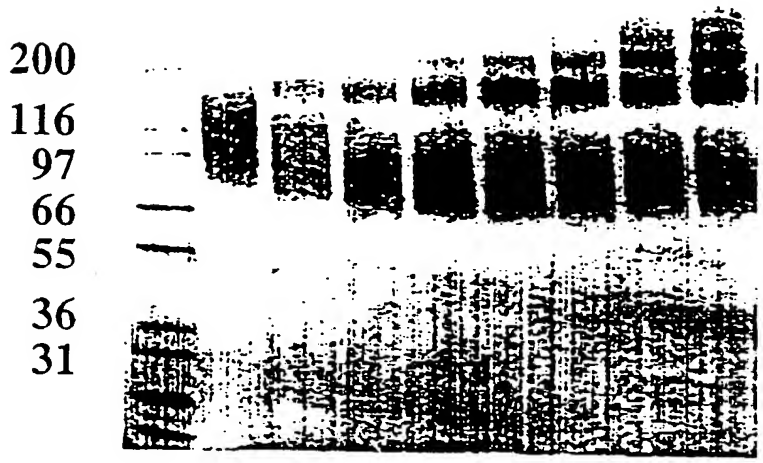

figure 11A

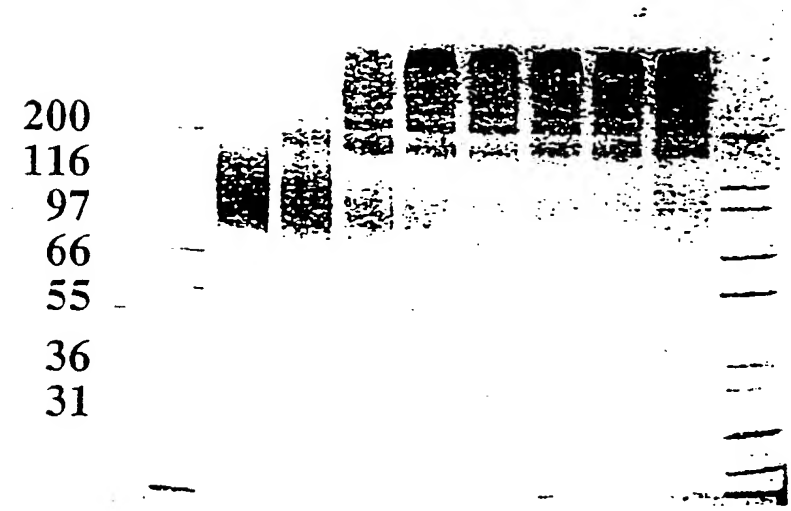

figure 11B

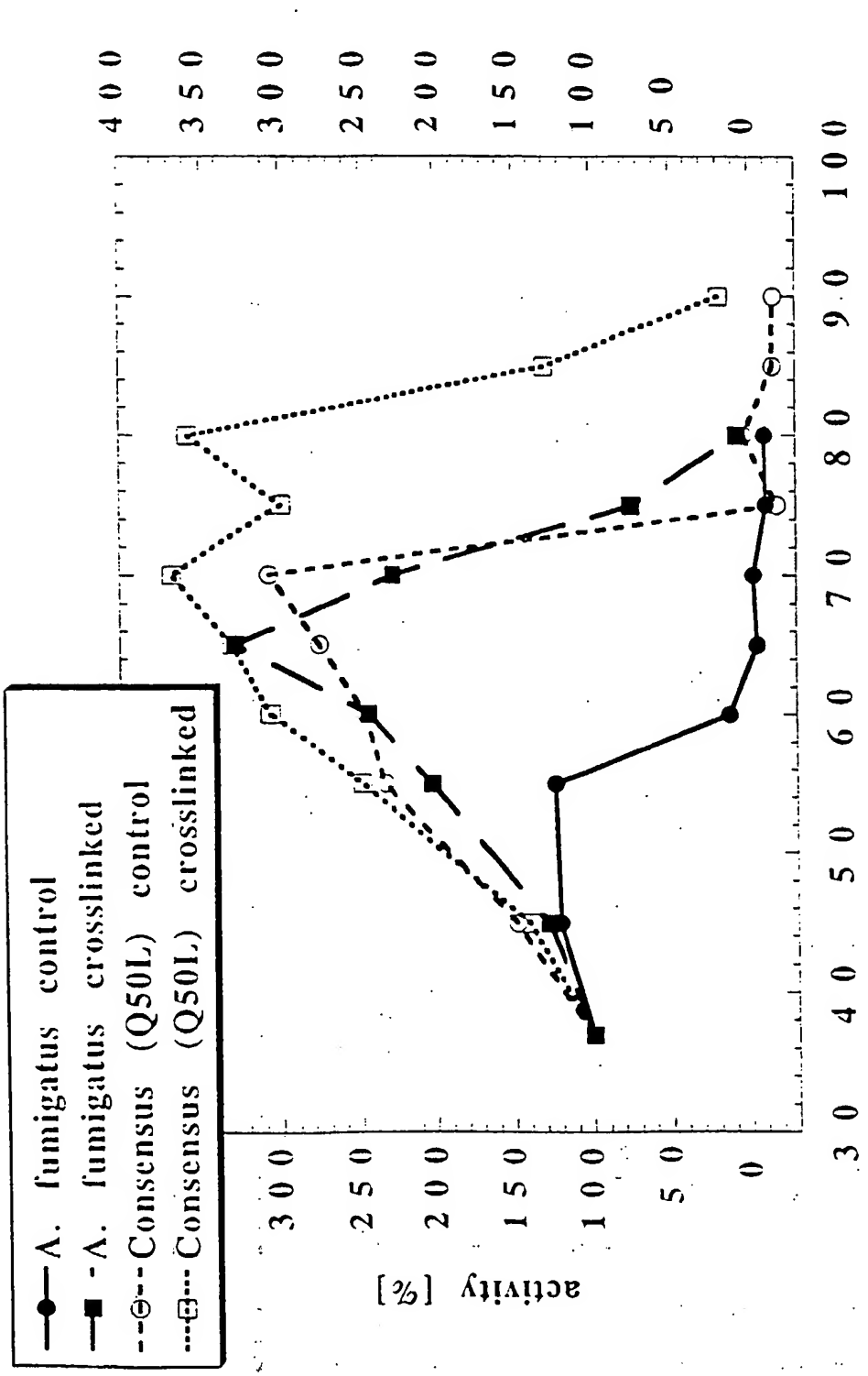

figure 12

Figure 13

```
                                    1
50
A. terreus 9A-1                     KhsDCNSVDh GYQCFPELSH kWGlYAPYFS
LQDESPFPlD VPEDChITFV
A. terreus cbs                      NhsDCTSVDr GYQCFPELSH kWGlYAPYFS
LQDESPFPlD VPDDChITFV
A. niger var. awamori               NqsTCDTVDQ GYQCFSETSH LWGQYAPFFS
LANESAISPD VPAGCrVTFA
A. niger T213                       NqsSCDTVDQ GYQCFSETSH LWGQYAPFFS
LANESVISPD VPAGCrVTFA
A. niger NRRL3135                   NqsSCDTVDQ GYQCFSETSH LWGQYAPFFS
LANESVISPE VPAGCrVTFA
A. fumigatus 13073                  GSkSCDTVDl GYQCsPATSH LWGQYSPFFS
LEDElSVSSK LPKDCrITLV
A. fumigatus 32722                  GSkSCDTVDl GYQCsPATSH LWGQYSPFFS
LEDElSVSSK LPKDCrITLV
A. fumigatus 58128                  GSkSCDTVDl GYQCsPATSH LWGQYSPFFS
LEDElSVSSK LPKDCrITLV
A. fumigatus 26906                  GSkSCDTVDl GYQCsPATSH LWGQYSPFFS
LEDElSVSSK LPKDCrITLV
A. fumigatus 32239                  GSkACDTVEl GYQCsPGTSH LWGQYSPFFS
LEDElSVSSD LPKDCrVTFV
E. nidulans                         QNHSCNTADG GYQCFPNVSH VWGQYSPYFS
IEQESAISeD VPHGCeVTFV
T. thermophilus                     DSHSCNTVEG GYQCrPEISH sWGQYSPFFS
LADQSEISPD VPQNCkITFV
M. thermophila                      ESRPCDTpDl GFQCgTAISH FWGQYSPYFS
VpSElDaS.. IPDDCeVTFA

Consensus                           NSHSCDTVDG GYQCFPEISH LWGQYSPYFS
LEDESAISPD VPDDC-VTFV
Consensus phytase                   NSHSCDTVDG GYQCFPEISH LWGQYSPYFS
LEDESAISPD VPDDCRVTFV


                                    51
100
A. terreus 9A-1                     QVLARHGARs PThSKtKAYA AtIAAIQKSA
TaFpGKYAFL QSYNYSLDSE
A. terreus cbs                      QVLARHGARs PTDSKtKAYA AtIAAIQKNA
TaLpGKYAFL KSYNYSMGSE
A. niger var. awamori               QVLSRHGARY PTESKgKkYS ALIEEIQQNV
TtFDGKYAFL KTYNYSLGAD
A. niger T213                       QVLSRHGARY PTESKgKkYS ALIEEIQQNV
TtFDGKYAFL KTYNYSLGAD
A. niger NRRL3135                   QVLSRHGARY PTDSKgKkYS ALIEEIQQNA
TtFDGKYAFL KTYNYSLGAD
A. fumigatus 13073                  QVLSRHGARY PTSSKsKkYK kLVTAIQaNA
TdFKGKFAFL KTYNYTLGAD
A. fumigatus 32722                  QVLSRHGARY PTSSKsKkYK kLVTAIQaNA
TdFKGKFAFL KTYNYTLGAD
A. fumigatus 58128                  QVLSRHGARY PTSSKsKkYK kLVTAIQaNA
TdFKGKFAFL KTYNYTLGAD
A. fumigatus 26906                  QVLSRHGARY PTSSKsKkYK kLVTAIQaNA
TdFKGKFAFL KTYNYTLGAD
A. fumigatus 32239                  QVLSRHGARY PTASKsKkYK kLVTAIQKNA
TeFKGKFAFL ETYNYTLGAD
E. nidulans                         QVLSRHGARY PTESKsKAYS GLIEAIQKNA
TsFwGQYAFL ESYNYTLGAD
```

T. thermophilus	QLLSRHGARY PTSSKtElYS QLISrIQKTA	
TaYKGyYAFL KDYrYqLGAN		
M. thermophila	QVLSRHGARa PTlKRaaSYv DLIDrIHhGA	
IsYgPgYEFL RTYDYTLGAD		
Consensus	QVLSRHGARY PTSSK-KAYS ALIEAIQKNA T-	
FKGKYAFL KTYNYTLGAD		
Consensus phytase	**QVLSRHGARY PTSSKSKAYS ALIEAIQKNA**	
TAFKGKYAFL KTYNYTLGAD		

101

150

A. terreus 9A-1	ELTPFGrNQL rDlGaQFYeR YNALTRhInP	
FVRATDASRV hESAEKFVEG		
A. terreus cbs	NLTPFGrNQL qDlGaQFYRR YDTLTRhInP	
FVRAADSSRV hESAEKFVEG		
A. niger var. *awamori*	DLTPFGEQEL VNSGIKFYQR YESLTRNIIP	
FIRSSGSSRV IASGEKFIEG		
A. niger T213	DLTPFGEQEL VNSGIKFYQR YESLTRNIIP	
FIRSSGSSRV IASGEKFIEG		
A. niger NRRL3135	DLTPFGEQEL VNSGIKFYQR YESLTRNIVP	
FIRSSGSSRV IASGKKFIEG		
A. fumigatus 13073	DLTPFGEQQL VNSGIKFYQR YKALARSVVP	
FIRASGSDRV IASGEKFIEG		
A. fumigatus 32722	DLTPFGEQQL VNSGIKFYQR YKALARSVVP	
FIRASGSDRV IASGEKFIEG		
A. fumigatus 58128	DLTPFGEQQL VNSGIKFYQR YKALARSVVP	
FIRASGSDRV IASGEKFIEG		
A. fumigatus 26906	DLTAFGEQQL VNSGIKFYQR YKALARSVVP	
FIRASGSDRV IASGEKFIEG		
A. fumigatus 32239	DLTPFGEQQM VNSGIKFYQK YKALAgSVVP	
FIRSSGSDRV IASGEKFIEG		
E. nidulans	DLTiFGENQM VDSGaKFYRR YKNLARKnTP	
FIRASGSDRV VASAEKFING		
T. thermophilus	DLTPFGENQM IQlGIKFYnH YKSLARNaVP	
FVRCSGSDRV IASGrlFIEG		
M. thermophila	ELTRtGQQQM VNSGIKFYRR YRALARKsIP	
FVRTAGqDRV VhSAENFTQG		
Consensus	DLTPFGENQM VNSGIKFYRR YKALARK-VP	
FVRASGSDRV IASAEKFIEG		
Consensus phytase	**DLTPFGENQM VNSGIKFYRR YKALARKIVP**	
FIRASGSDRV IASAEKFIEG		

151

200

A. terreus 9A-1 FQTARqDDHh ANpHQPSPrV DVaIPEGSAY
NNTLEHSlCT AFES...STV
A. terreus cbs FQNARqGDPh ANpHQPSPrV DVVIPEGTAY
NNTLEHSICT AFEA...STV
A. niger var. *awamori* FQSTKLkDPr AqpgQSSPkI DVVISEASSs
NNTLDPGTCT VFED...SEL
A. niger T213 FQSTKLkDPr AqpgQSSPkI DVVISEASSs
NNTLDPGTCT VFED...SEL
A. niger NRRL3135 FQSTKLkDPr AqpgQSSPkI DVVISEASSs
NNTLDPGTCT VFED...SEL
A. fumigatus 13073 FQqAKLADPG A.TNRAAPAI SVIIPESETF
NNTLDHGVCT kFEA...SQL
A. fumigatus 32722 FQqAKLADPG A.TNRAAPAI SVIIPESETF
NNTLDHGVCT kFEA...SQL
A. fumigatus 58128 FQqAKLADPG A.TNRAAPAI SVIIPESETF
NNTLDHGVCT kFEA...SQL
A. fumigatus 26906 FQqAKLADPG A.TNRAAPAI SVIIPESETF
NNTLDHGVCT kFEA...SQL
A. fumigatus 32239 FQqANVADPG A.TNRAAPVI SVIIPESETY
NNTLDHSVCT NFEA...SEL
E. nidulans FRKAQLhDHG S..gQATPVV NVIIPEiDGF
NNTLDHSTCV SFEN...DEr
T. thermophilus FQSAKVlDPh SDkHDAPPTI NVIIeEGPSY
NNTLDtGSCP VFED...SSg
M. thermophila FHSAlLADRG STvRPTlPyd mVVIPETAGa
NNTLHNDlCT AFEEgpySTI

Consensus FQSAKLADPG S-PHQASPVI NVIIPEGSGY
NNTLDHGTCT AFED---SEL
Consensus phytase FQSAKLADPG SQPHQASPVI DVIIPEGSGY
NNTLDHGTCT AFED...SEL

201

250

A. terreus 9A-1 GDDAvANFTA VFAPAIaQRL EADLPGVqLS
TDDVVnLMAM CPFETVSlTD
A. terreus cbs GDAAADNFTA VFAPAIakRL EADLPGVqLS
ADDVVnLMAM CPFETVSlTD
A. niger var. *awamori* ADTVEANFTA TFAPSIRQRL ENDLSGVTLT
DTEVTyLMDM CSFDTIStST
A. niger T213 ADTVEANFTA TFAPSIRQRL ENDLSGVTLT
DTEVTyLMDM CSFDTIStST
A. niger NRRL3135 ADTVEANFTA TFVPSIRQRL ENDLSGVTLT
DTEVTyLMDM CSFDTIStST
A. fumigatus 13073 GDEVAANFTA lFAPDIRARa EkHLPGVTLT
DEDVVsLMDM CSFDTVARTS
A. fumigatus 32722 GDEVAANFTA lFAPDIRARa EkHLPGVTLT
DEDVVsLMDM CSFDTVARTS
A. fumigatus 58128 GDEVAANFTA lFAPDIRARa EkHLPGVTLT
DEDVVsLMDM CSFDTVARTS
A. fumigatus 26906 GDEVAANFTA lFAPDIRARa KkHLPGVTLT
DEDVVsLMDM CSFDTVARTS
A. fumigatus 32239 GDEVEANFTA lFAPAIRARI EkHLPGVqLT
DDDVVsLMDM CSFDTVARTA
E. nidulans ADEiEANFTA IMGPPIRkRL ENDLPGIKLT
NENVIyLMDM CSFDTMARTA
T. thermophilus GHDAQEKFAk qFAPAIlEKI KDHLPGVDLA
vSDVpyLMDL CPFETLARNh

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M. thermophila          GDDAQDTYlS TFAGPItARV NANLPGANLT
DADTVaLMDL CPFETVAsSS

Consensus               GDDAEANFTA TFAPAIRARL EADLPGVTLT DEDVV-
LMDM CPFETVARTS
Consensus phytase       GDDVEANFTA LFAPAIRARL EADLPGVTLT
DEDVVYLMDM CPFETVARTS

                        251
300
A. terreus 9A-1         ........ ...DAhTLSPFC DLFTAtEWtq
YNYLlSLDKY YGYGGGNPLG
A. terreus cbs          ........ ...DAhTLSPFC DLFTAaEWtq
YNYLlSLDKY YGYGGGNPLG
A. niger var. awamori   ........ ...vDTKLSPFC DLFTHdEWih
YDYLQSLkKY YGHGAGNPLG
A. niger T213           ........ ...vDTKLSPFC DLFTHdEWih
YDYLRSLkKY YGHGAGNPLG
A. niger NRRL3135       ........ ...vDTKLSPFC DLFTHdEWin
YDYLQSLkKY YGHGAGNPLG
A. fumigatus 13073      ........ ...DASQLSPFC QLFTHnEWkk
YNYLQSLGKY YGYGAGNPLG
A. fumigatus 32722      ........ ...DASQLSPFC QLFTHnEWkk
YNYLQSLGKY YGYGAGNPLG
A. fumigatus 58128      ........ ...DASQLSPFC QLFTHnEWkk
YNYLQSLGKY YGYGAGNPLG
A. fumigatus 26906      ........ ...DASQLSPFC QLFTHnEWkk
YNYLQSLGKY YGYGAGNPLG
A. fumigatus 32239      ........ ...DASELSPFC AIFTHnEWkk
YDYLQSLGKY YGYGAGNPLG
E. nidulans             ........ ...HGTELSPFC AIFTEkEWlq
YDYLQSLSKY YGYGAGSPLG
T. thermophilus         ........ ...TDT.LSPFC ALsTQeEWqa
YDYYQSLGKY YGnGGGNPLG
M. thermophila          sdpatadagg gNGrpLSPFC rLFSEsEWra
YDYLQSVGKW YGYGPGNPLG

Consensus               ---------- -DATELSPFC ALFTE-EW--
YDYLQSLGKY YGYGAGNPLG
Consensus phytase       .......... .DATELSPFC ALFTHDEWRQ
YDYLQSLGKY YGYGAGNPLG
```

```
                                301
350
A. terreus 9A-1          PVQGVGWaNE LMARLTRAPV HDHTCVNNTL
DASPATFPLN ATLYADFSHD
A. terreus cbs           PVQGVGWaNE LIARLTRSPV HDHTCVNNTL
DANPATFPLN ATLYADFSHD
A. niger var. awamori    PTQGVGYaNE LIARLTHSPV HDDTSSNHTL
DSNPATFPLN STLYADFSHD
A. niger T213            PTQGVGYaNE LIARLTHSPV HDDTSSNHTL
DSNPATFPLN STLYADFSHD
A. niger NRRL3135        PTQGVGYaNE LIARLTHSPV HDDTSSNHTL
DSSPATFPLN STLYADFSHD
A. fumigatus 13073       PAQGIGFtNE LIARLTRSPV QDHTSTNsTL
vSNPATFPLN ATMYVDFSHD
A. fumigatus 32722       PAQGIGFtNE LIARLTRSPV QDHTSTNsTL
vSNPATFPLN ATMYVDFSHD
A. fumigatus 58128       PAQGIGFtNE LIARLTRSPV QDHTSTNsTL
vSNPATFPLN ATMYVDFSHD
A. fumigatus 26906       PAQGIGFtNE LIARLTRSPV QDHTSTNsTL
vSNPATFPLN ATMYVDFSHD
A. fumigatus 32239       PAQGIGFtNE LIARLTNSPV QDHTSTNsTL
DSDPATFPLN ATIYVDFSHD
E. nidulans              PAQGIGFtNE LIARLTQSPV QDNTSTNHTL
DSNPATFPLD rKLYADFSHD
T. thermophilus          PAQGVGFvNE LIARMTHSPV QDYTTVNHTL
DSNPATFPLN ATLYADFSHD
M. thermophila           PTQGVGFvNE LLARLAgvPV RDgTSTNRTL
DGDPrTFPLG rPLYADFSHD

Consensus                PAQGVGF-NE LIARLTHSPV QDHTSTNHTL
DSNPATFPLN ATLYADFSHD
Consensus phytase        PAQGVGFANE LIARLTRSPV QDHTSTNHTL
DSNPATFPLN ATLYADFSHD


                                351
400
A. terreus 9A-1          SNLVSIFWAL GLYNGTAPLS qTSVESVSQT
DGYAAAWTVP FAARAYVEMM
A. terreus cbs           SNLVSIFWAL GLYNGTkPLS qTTVEDITrT
DGYAAAWTVP FAARAYIEMM
A. niger var. awamori    NGIISILFAL GLYNGTkPLS TTTVENITQT
DGFSSAWTVP FASRlYVEMM
A. niger T213            NGIISILFAL GLYNGTkPLS TTTVENITQT
DGFSSAWTVP FASRlYVEMM
A. niger NRRL3135        NGIISILFAL GLYNGTkPLS TTTVENITQT
DGFSSAWTVP FASRlYVEMM
A. fumigatus 13073       NSMVSIFFAL GLYNGTEPLS rTSVESaKEl
DGYSASWVVP FGARAYFEtM
A. fumigatus 32722       NSMVSIFFAL GLYNGTGPLS rTSVESaKEl
DGYSASWVVP FGARAYFEtM
A. fumigatus 58128       NSMVSIFFAL GLYNGTEPLS rTSVESaKEl
DGYSASWVVP FGARAYFEtM
A. fumigatus 26906       NSMVSIFFAL GLYNGTEPLS rTSVESaKEl
DGYSASWVVP FGARAYFEtM
A. fumigatus 32239       NGMIPIFFAM GLYNGTEPLS qTSeESTKES
NGYSASWAVP FGARAYFEtM
E. nidulans              NSMISIFFAM GLYNGTQPLS mDSVESIQEm
DGYAASWTVP FGARAYFELM
T. thermophilus          NTMTSIFaAL GLYNGTAkLS TTEIKSIEET
DGYSAAWTVP FGGRAYIEMM
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M. thermophila          NDMMGVLgAL GaYDGVPPLD KTArrDpEEl
GGYAASWAVP FAARiYVEKM

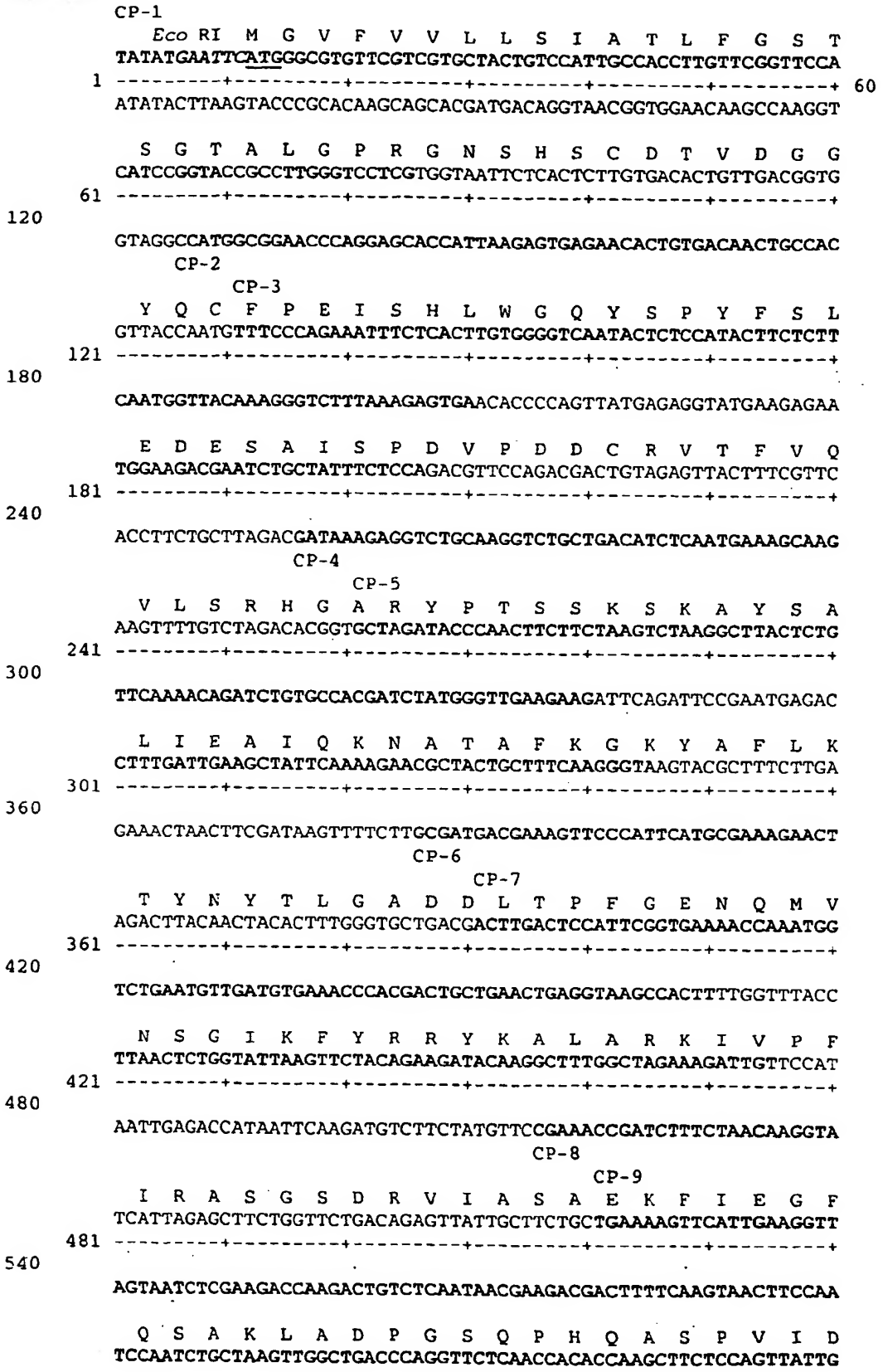
Consensus               NSMISIFFAL GLYNGTAPLS TTSVESIEET
DGYAASWTVP FGARAYVEMM
Consensus phytase       NSMISIFFAL GLYNGTAPLS TTSVESIEET
DGYSASWTVP FGARAYVEMM

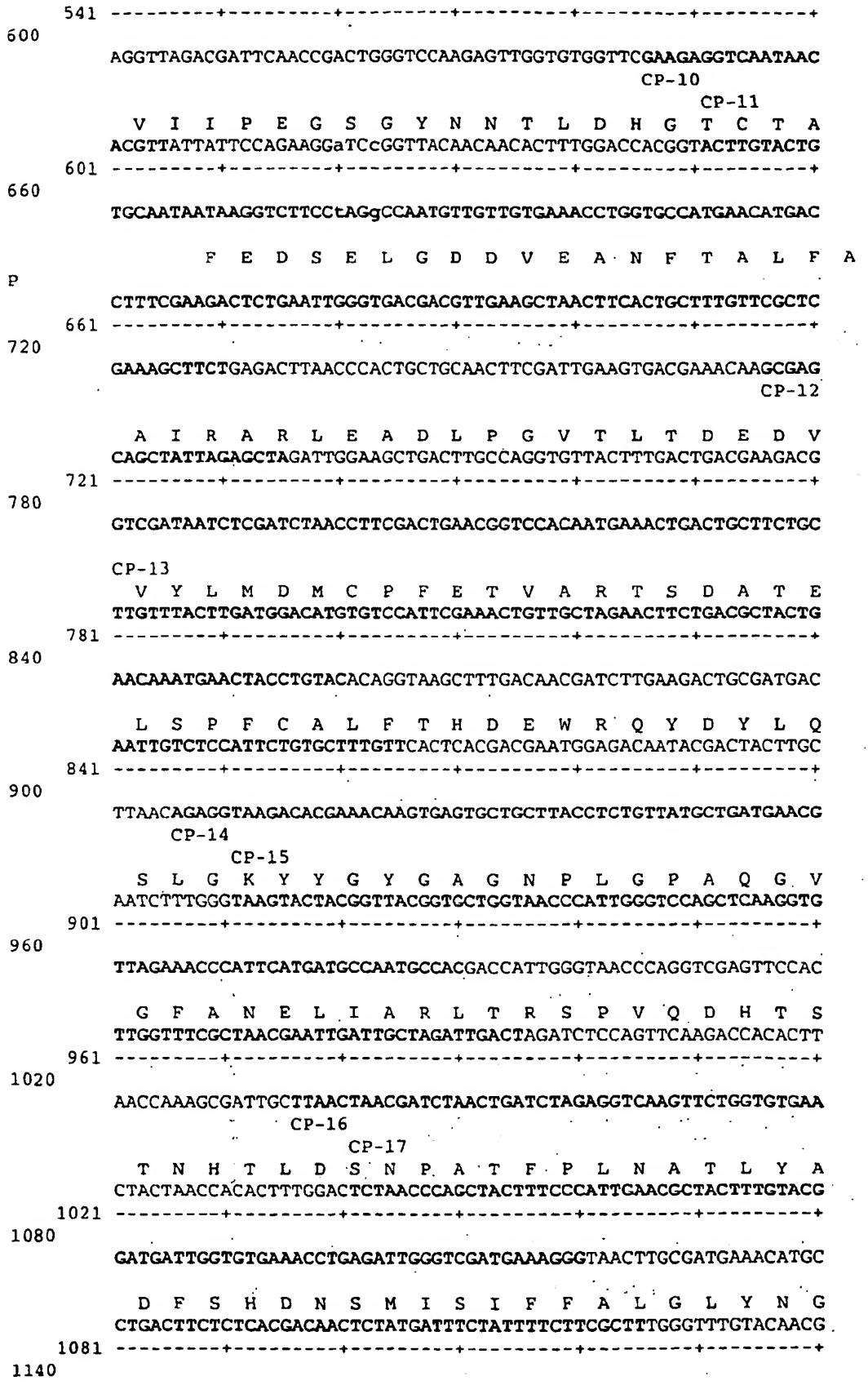
                        401
450
A. terreus 9A-1         QC..... .....RAEKE PLVRVLVNDR
VMPLHGCPTD KLGRCKrDAF
A. terreus cbs          QC..... .....RAEKQ PLVRVLVNDR
VMPLHGCAVD NLGRCKrDDF
A. niger var. awamori   QC..... .....QAEQE PLVRVLVNDR
VVPLHGCPID aLGRCTrDSF
A. niger T213           QC..... .....QAEQE PLVRVLVNDR
VVPLHGCPID aLGRCTrDSF
A. niger NRRL3135       QC..... .....QAEQE PLVRVLVNDR
VVPLHGCPVD aLGRCTrDSF
A. fumigatus 13073      QC..... .....KSEKE PLVRALINDR
VVPLHGCDVD KLGRCKLNDF
A. fumigatus 32722      QC..... .....KSEKE PLVRALINDR
VVPLHGCDVD KLGRCKLNDF
A. fumigatus 58128      QC..... .....KSEKE SLVRALINDR
VVPLHGCDVD KLGRCKLNDF
A. fumigatus 26906      QC..... .....KSEKE PLVRALINDR
VVPLHGCDVD KLGRCKLNDF
A. fumigatus 32239      QC..... .....KSEKE PLVRALINDR
VVPLHGCAVD KLGRCKLKDF
E. nidulans             QC..... .....E.KKE PLVRVLVNDR
VVPLHGCAVD KFGRCTLDDW
T. thermophilus         QC..... .....DDSDE PVVRVLVNDR
VVPLHGCEVD SLGRCKrDDF
M. thermophila          RCsgggggg ggegrQEKDE eMVRVLVNDR
VMTLkGCGAD ErGMCTLErF

Consensus               QC-------- -----QAEKE PLVRVLVNDR
VVPLHGCAVD KLGRCKLDDF
Consensus phytase       QC..... .....QAEKE PLVRVLVNDR
VVPLHGCAVD KLGRCKRDDF

                      451
471
A. terreus 9A-1         VAGLSFAQAG GNWADCF~~~ ~
A. terreus cbs          VEGLSFARAG
GNWAECF~~~ ~
A. niger var. awamori   VrGLSFARSG GDWAECsA~~ ~
A. niger T213           VrGLSFARSG GDWAECFA~~ ~
A. niger NRRL3135       VrGLSFARSG
GDWAECFA~~ ~
A. fumigatus 13073      VKGLSWARSG GNWGECFS~~ ~
A. fumigatus 32722      VKGLSWARSG GNWGECFS~~ ~
A. fumigatus 58128      VKGLSWARSG GNWGECFS~~ ~
A. fumigatus 26906      VKGLSWARSG GNWGECFS~~ ~
A. fumigatus 32239      VKGLSWARSG
GNSEQSFS~~ ~
E. nidulans             VEGLNFARSG GNWkTCFTl~ ~
T. thermophilus         VrGLSFARqG GNWEGCYAas e
M. thermophila          IESMAFARGN GKWDlCFA~~ ~
Consensus               VEGLSFARSG GNWAECFA~~ ~
Consensus phytase       VEGLSFARSG GNWAECFA.. .
```

Figure 14

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CP-11
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601
660
TGCAATAATAAGGTCTTCCtAGgCCAATGTTGTTGTGAAACCTGGTGCCATGAACATGAC
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P
CTTTCGAAGACTCTGAATTGGGTGACGACGTTGAAGCTAACTTCACTGCTTTGTTCGCTC
661
720
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CP-12
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721
780
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781
840
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L S P F C A L F T H D E W R Q Y D Y L Q
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841
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S L G K Y Y G Y G A G N P L G P A Q G V
AATCTTTGGGTAAGTACTACGGTTACGGTGCTGGTAACCCATTGGGTCCAGCTCAAGGTG
901
960
TTAGAAACCCATTCATGATGCCAATGCCACGACCATTGGGTAACCCAGGTCGAGTTCCAC
G F A N E L I A R L T R S P V Q D H T S
TTGGTTTCGCTAACGAATTGATTGCTAGATTGACTAGATCTCCAGTTCAAGACCACACTT
961
1020
AACCAAAGCGATTGCTTAACTAACGATCTAACTGATCTAGAGGTCAAGTTCTGGTGTGAA
CP-16
CP-17
T N H T L D S N P A T F P L N A T L Y A
CTACTAACCACACTTTGGACTCTAACCCAGCTACTTTCCCATTGAACGCTACTTTGTACG
1021
1080
GATGATTGGTGTGAAACCTGAGATTGGGTCGATGAAAGGGTAACTTGCGATGAAACATGC
D F S H D N S M I S I F F A L G L Y N G
CTGACTTCTCTCACGACAACTCTATGATTTCTATTTTCTTCGCTTTGGGTTTGTACAACG
1081
1140

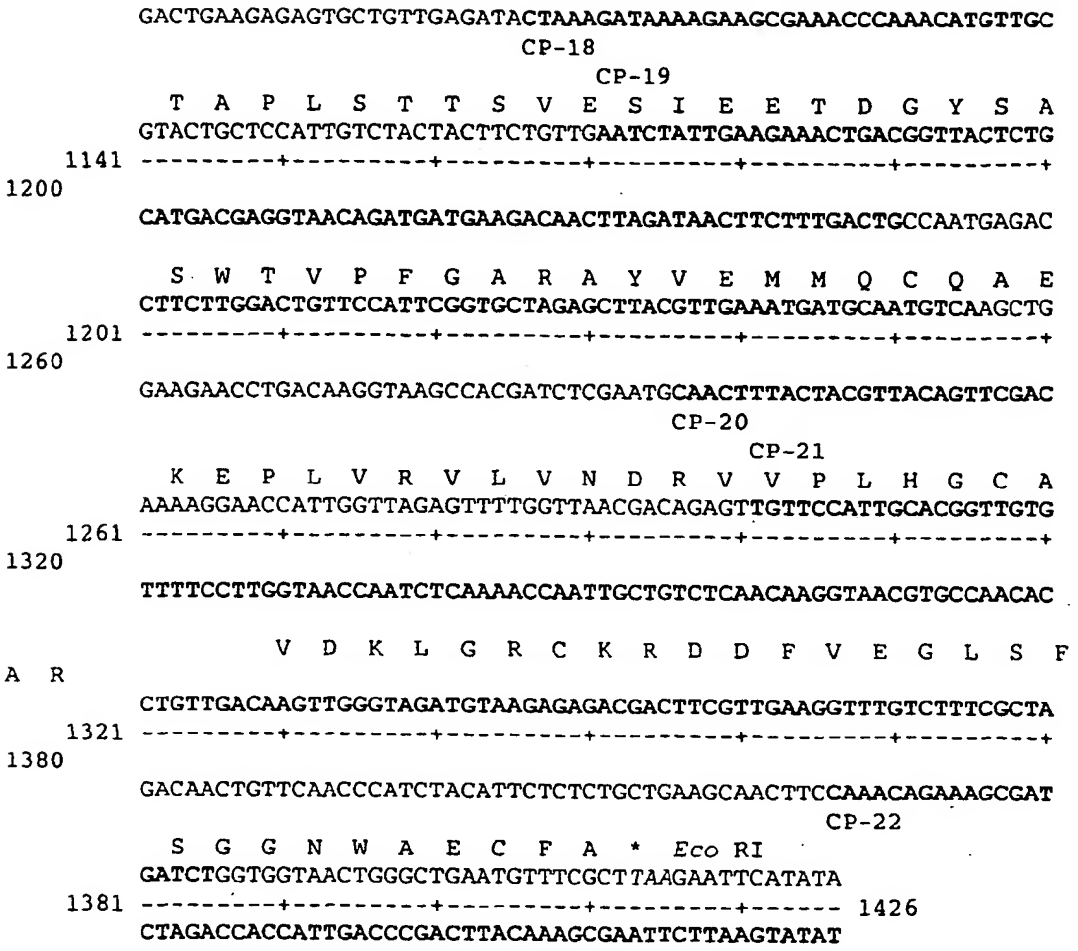
GACTGAAGAGAGTGCTGTTGAGATACTAAAGATAAAAGAAGCGAAACCCAAACATGTTGC
CP-18
CP-19
T A P L S T T S V E S I E E T D G Y S A
GTACTGCTCCATTGTCTACTACTTCTGTTGAATCTATTGAAGAAACTGACGGTTACTCTG
1141 ---------+---------+---------+---------+---------+---------+
1200
CATGACGAGGTAACAGATGATGAAGACAACTTAGATAACTTCTTTGACTGCCAATGAGAC
S W T V P F G A R A Y V E M M Q C Q A E
CTTCTTGGACTGTTCCATTCGGTGCTAGAGCTTACGTTGAAATGATGCAATGTCAAGCTG
1201 ---------+---------+---------+---------+---------+---------+
1260
GAAGAACCTGACAAGGTAAGCCACGATCTCGAATGCAACTTTACTACGTTACAGTTCGAC
CP-20
CP-21
K E P L V R V L V N D R V V P L H G C A
AAAAGGAACCATTGGTTAGAGTTTTGGTTAACGACAGAGTTGTTCCATTGCACGGTTGTG
1261 ---------+---------+---------+---------+---------+---------+
1320
TTTTCCTTGGTAACCAATCTCAAAACCAATTGCTGTCTCAACAAGGTAACGTGCCAACAC
V D K L G R C K R D D F V E G L S F
A R
CTGTTGACAAGTTGGGTAGATGTAAGAGAGACGACTTCGTTGAAGGTTTGTCTTTCGCTA
1321 ---------+---------+---------+---------+---------+---------+
1380
GACAACTGTTCAACCCATCTACATTCTCTCTGCTGAAGCAACTTCCAAACAGAAAGCGAT
CP-22
S G G N W A E C F A * Eco RI
GATCTGGTGGTAACTGGGCTGAATGTTTCGCTTAAGAATTCATATA
1381 ---------+---------+---------+---------+------ 1426
CTAGACCACCATTGACCCGACTTACAAAGCGAATTCTTAAGTATAT

Figure 15

```
                    1
50
P. involutus (phyA1) SvP.KnTAPt FPIPeseQrn WSPYSPYFPL AeYkAPPAGC
QInQVNIIQR
P. involutus (phyA2) SvP.RniAPK FSIPeseQrn WSPYSPYFPL AeYkAPPAGC
EInQVNIIQR
T. pubescens         hiPlRdTSAc LdVTrDvQqs WSmYSPYFPa AtYvAPPASC
QInQVHIIQR
A. pediades          GgvvQaTfvQ pfFPpQiQds WAAYTPYYPV qaYtPPPkDC
KItQVNIIQR
P. lycii             StQfsfvAAQ LPIPaQntsn WGPYdPFFPV EpYaAPPEGC
tVtQVNLIQR

Basidio              S-P-R-TAAQ LPIP-Q-Q-- WSPYSPYFPV A-Y-APPAGC QI-
QVNIIQR

                    51
100
P. involutus (phyA1) HGARFPTSGA TTRIKAGLTK LQGvqnfTDA KFNFIkSfkY
dLGnsDLVPF
P. involutus (phyA2) HGARFPTSGA ATRIKAGLSK LQSvqnfTDP KFDFIkSfTY
dLGtsDLVPF
T. pubescens         HGARFPTSGA AkRIQTAVAK LKAAsnyTDP lLAFVtNyTY
sLGqDsLVeL
A. pediades          HGARFPTSGA GTRIQAAVkK LQSAktyTDP RLDFLtNyTY
tLGhDDLVPF
P. lycii             HGARWPTSGA rSRqvAAVAK IQmArpfTDP KYEFLnDfvY
kFGvADLLPF

Basidio              HGARFPTSGA ATRIQAAVAK LQSA---TDP KLDFL-N-TY -LG-
DDLVPF

                    101
150
P. involutus (phyA1) GAaQSfDAGQ EAFARYSkLV SkNNLPFIRA dGSDRVVDSA
TNWTAGFAsA
P. involutus (phyA2) GAaQSfDAGl EvFARYSkLV SsDNLPFIRS dGSDRVVDTA
TNWTAGFAsA
T. pubescens         GAtQSSEAGQ EAFTRYSsLV SaDELPFVRA SGSDRVVATA
nNWTAGFAlA
A. pediades          GAlQSSQAGE ETFqRYSfLV SkENLPFVRA SSSNRVVDSA
TNWTEGFSaA
P. lycii             GAnQShQTGt DmYTRYStLf egGDVPFVRA AGdQRVVDSS
TNWTAGFGdA

Basidio              GA-QSSQAGQ EAFTRYS-LV S-DNLPFVRA SGSDRVVDSA
TNWTAGFA-A

                    151
200
P. involutus (phyA1) ShNTvqPkLn LILPQtGNDT LEDNMCPaAG DSDPQvNaWL
AVafPSITAR
P. involutus (phyA2) SrNAiqPkLd LILPQtGNDT LEDNMCPaAG ESDPQvDaWL
AsafPSVTAQ
T. pubescens         SsNSitPvLs VIISEaGNDT LDDNMCPaAG DSDPQvNqWL
AqFAPPMTAR
A. pediades          ShHvlnPiLf VILSEslNDT LDDaMCPnAG sSDPQtGiWt
SIYGTPIAnR
```

```
P. lycii                SgETvlPtLq VVLqEeGNcT LcNNMCPnEv DGDest.tWL
GVFAPnITAR

Basidio                 S-NT--P-L- VILSE-GNDT LDDNMCP-AG DSDPQ-N-WL
AVFAPPITAR


                                201
250
P. involutus (phyA1)    LNAAAPSvNL TDtDAfNLvs LCAFlTVSkE kkSdFCtLFE
giPGsFeAFa
P. involutus (phyA2)    LNAAAPGANL TDaDAfNLvs LCPFmTVSkE qkSdFCtLFE
giPGsFeAFa
T. pubescens            LNAGAPGANL TDtDTyNLlt LCPFETVAtE rrSeFCDIYE
elQAE.dAFa
A. pediades             LNqqAPGANI TAaDvsNLip LCAFETIvkE tpSpFCNLF.
.tPEEFaqFe
P. lycii                LNAAAPSANL SDsDAltLmd MCPFDTLSsG naSpFCDLF.
.tAEEYvSYe

Basidio                 LNAAAPGANL TD-DA-NL-- LCPFETVS-E --S-FCDLFE --PEEF-
AF-


                                251
300
P. involutus (phyA1)    YgGDLDKFYG TGYGQeLGPV QGVGYVNELI ARLTnsAVRD
NTQTNRTLDA
P. involutus (phyA2)    YaGDLDKFYG TGYGQALGPV QGVGYINELL ARLTnsAVnD
NTQTNRTLDA
T. pubescens            YnADLDKFYG TGYGQPLGPV QGVGYINELI ARLTaQnVsD
HTQTNsTLDS
A. pediades             YfGDLDKFYG TGYGQPLGPV QGVGYINELL ARLTemPVRD
NTQTNRTLDS
P. lycii                YyyDLDKYYG TGpGNALGPV QGVGYVNELL ARLTgQAVRD
ETQTNRTLDS

Basidio                 Y-GDLDKFYG TGYGQPLGPV QGVGYINELL ARLT-QAVRD
NTQTNRTLDS


                                301
350
P. involutus (phyA1)    SPvTFPLNKT FYADFSHDNl MVAVFSAMGL FrQPAPLsTS
vPNPwRTWrT
P. involutus (phyA2)    APdTFPLNKT MYADFSHDNl MVAVFSAMGL FrQSAPLsTS
tPDPNRTWLT
T. pubescens            SPeTFPLNRT LYADFSHDNQ MVAIFSAMGL FNQSAPLDPT
tPDPaRTFLv
A. pediades             SPlTFPLDRS IYADLSHDNQ MIAIFSAMGL FNQSSPLDPS
fPNPKRTWVT
P. lycii                dPaTFPLNRT FYADFSHDNt MVPIFAALGL FNaTA.LDPl
kPDeNRlWVd

Basidio                 SP-TFPLNRT FYADFSHDNQ MVAIFSAMGL FNQSAPLDPS -
PDPNRTWVT


                                351
400
P. involutus (phyA1)    SsLVPFSGRM VVERLsC..f GT..... .....tkV
RVLVQDqVQP
```

```
P. involutus (phyA2) SsVVPFSARM aVERLsC..a GT..... .......tkV
RVLVQDqVQP
T. pubescens         kKIVPFSARM VVERLdC..g GA..... .......qsV
RLLVNDAVQP
A. pediades          SRLtPFSARM VtERL1Cqrd GTgsggpsri mrngnvqtfV
RILVNDALQP
P. lycii             SKLVPFSGHM tVEKLaC... .......... ....sgkeaV
RVLVNDAVQP

Basidio              SKLVPFSARM VVERL-C--- GT-------- ---------V
RVLVNDAVQP


                     401                                       441
P. involutus (phyA1) LEFCGGDrNG lCTLAkFVES QtFARsDGaG DFEKCFATSa -
P. involutus (phyA2) LEFCGGDqDG lCALDkFVES QaYARsGGaG DFEKCLATTv -
T. pubescens         LAFCGADtsG vCTLDAFVES QaYARNDGEG DFEKCFAT-- -
A. pediades          LKFCGGDmDS lCTLEAFVES QkYAREDGQG DFEKCFD--- -
P. lycii             LEFCGG.vDG vCeLsAFVES QtYARENGQG DFAKCgfvPs e

Basidio              LEFCGGD-DG -CTLDAFVES Q-YAREDGQG DFEKCFATP- -
```

Figure 16

```
                                1
50
   A. terreus 9a1               KhsdCNSVDh GYQCfPELSH kWGlYAPYFS LqDESPFPlD
VPeDCHITFV
   A. terreus cbs               NhsdCtSVDr GYQCfPELSH kWGlYAPYFS LqDESPFPlD
VPdDCHITFV
   A. niger var. awamori        NqsTCDTVDq GYQCfSEtSH LWGQYAPFFS LANESAISPD
VPaGCRVTFa
   A. niger NRRL3135            NqsSCDTVDq GYQCfSEtSH LWGQYAPFFS LANESvISPE
VPaGCRVTFa
   A. fumigatus 13073           GSkSCDTVDl GYQCsPAtSH LWGQYSPFFS LEDElSVSSK
LPkDCRITLV
   A. fumigatus 32722           GSkSCDTVDl GYQCsPAtSH LWGQYSPFFS LEDElSVSSK
LPkDCRITLV
   A. fumigatus 58128           GSkSCDTVDl GYQCsPAtSH LWGQYSPFFS LEDElSVSSK
LPkDCRITLV
   A. fumigatus 26906           GSkSCDTVDl GYQCsPAtSH LWGQYSPFFS LEDElSVSSK
LPkDCRITLV
   A. fumigatus 32239           GSkACDTVEl GYQCsPGtSH LWGQYSPFFS LEDElSVSSD
LPkDCRVTFV
   E. nidulans                  QNHSCNTaDG GYQCfPNVSH VWGQYSPYFS IEQESAISeD
VPhGCeVTFV
   T. thermophilus              DSHSCNTVEG GYQCrPEISH sWGQYSPFFS LADQSEISPD
VPqNCKITFV
   T. lanuginosa                ~~~~~~~~~~ ~~~~nvDIAR hWGQYSPFFS LAEvSEISPA
VPkGCRVeFV
   M. thermophila               ESRPCDTpDl GFQCgTAISH FWGQYSPYFS VPsElDaS..
IPdDCeVTFa
   Basidio                      xSxPxrxtAA qLPipxQxqx xWSPYSPYFP VAxyxA....
pPaGCQIxqV

             Consensus          NSHSCDTVDG GYQC-PEISH LWGQYSPFFS LADESAISPD VP-
GCRVTFV
                 Fcp10          NSHSCDTVDG GYQCFPEISH LWGQYSPFFS LADESAISPD
VPKGCRVTFV


                                51
100
   A. terreus 9a1               QVLARHGARs PThSKTKaYA AtIaAIQKSA TaFpGKYAFL
QSYNYSLDSE
   A. terreus cbs               QVLARHGARs PTdSKTKaYA AtIaAIQKNA TaLpGKYAFL
KSYNYSMGSE
   A. niger var. awamori        QVLSRHGARY PTeSKGKKYS ALIeEIQQNv TtFDGKYAFL
KTYNYSLGAD
   A. niger NRRL3135            QVLSRHGARY PTdSKGKKYS ALIeEIQQNA TtFDGKYAFL
KTYNYSLGAD
   A. fumigatus 13073           QVLSRHGARY PTSSKSKKYk kLVtAIQaNA TdFKGKFAFL
KTYNYTLGAD
   A. fumigatus 32722           QVLSRHGARY PTSSKSKKYk kLVtAIQaNA TdFKGKFAFL
KTYNYTLGAD
   A. fumigatus 58128           QVLSRHGARY PTSSKSKKYk kLVtAIQaNA TdFKGKFAFL
KTYNYTLGAD
   A. fumigatus 26906           QVLSRHGARY PTSSKSKKYk kLVtAIQaNA TdFKGKFAFL
KTYNYTLGAD
   A. fumigatus 32239           QVLSRHGARY PTASKSKKYk kLVtAIQKNA TeFKGKFAFL
ETYNYTLGAD
   E. nidulans                  QVLSRHGARY PTeSKSKaYS GLIeAIQKNA TsFwGQYAFL
ESYNYTLGAD
```

```
   T. thermophilus        QLLSRHGARY PTSSKTElYS qLIsrIQKtA TaYKGyYAFL
KdYrYqLGAN
   T. lanuginosa          QVLSRHGARY PTAhKSEvYA ELLqrIQDtA TeFKGDFAFL
RdYaYhLGAD
   M. thermophila         QVLSRHGARa PTlkRAasYv DLIdrIHhGA isYgPgYEFL
RTYDYTLGAD
   Basidio                NIIqRHGARF PTSGaAtRiq AaVakLQsax xxtDPKLDFL
xnxtYxLGxD

            Consensus     QVLSRHGARY PTSSKSKKYS ALI-AIQKNA T-FKGKYAFL
KTYNYTLGAD
                 Fcp10    QVLSRHGARY PTSSKSKKYS ALIEAIQKNA TAFKGKYAFL
KTYNYTLGAD
```

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                          101
150
   A. terreus 9a1         ELTPFGrNQL rDlGaQFYeR YNAL.TRhIn PFVRATDAsR
VhESAEKFVE
   A. terreus cbs         NLTPFGrNQL qDlGaQFYRR YDTL.TRhIn PFVRAADSsR
VhESAEKFVE
   A. niger var. awamori  DLTPFGEQEL VNSGIKFYQR YESL.TRnII PFIRSSGSsR
VIASGEKFIE
   A. niger NRRL3135      DLTPFGEQEL VNSGIKFYQR YESL.TRnIV PFIRSSGSsR
VIASGKKFIE
   A. fumigatus 13073     DLTPFGEQQL VNSGIKFYQR YKAL.ARsVV PFIRASGSDR
VIASGEKFIE
   A. fumigatus 32722     DLTPFGEQQL VNSGIKFYQR YKAL.ARsVV PFIRASGSDR
VIASGEKFIE
   A. fumigatus 58128     DLTPFGEQQL VNSGIKFYQR YKAL.ARsVV PFIRASGSDR
VIASGEKFIE
   A. fumigatus 26906     DLTAFGEQQL VNSGIKFYQR YKAL.ARsVV PFIRASGSDR
VIASGEKFIE
   A. fumigatus 32239     DLTPFGEQQM VNSGIKFYQK YKAL.AgsVV PFIRSSGSDR
VIASGEKFIE
   E. nidulans            DLTiFGENQM VDSGaKFYRR YKnL.ARknt PFIRASGSDR
VVASAEKFIN
   T. thermophilus        DLTPFGENQM IQlGIKFYnH YKSL.ARnaV PFVRCSGSDR
VIASGrlFIE
   T. lanuginosa          NLTRFGEEQM MESGrQFYHR YREq.AReIV PFVRAAGSAR
VIASAEfFnr
   M. thermophila         ELTRtGQQQM VNSGIKFYRR YRAL.ARksI PFVRTAGqDR
VVhSAENFtQ
   Basidio                DLvPFGAxQs sQAGqEaFtR YsxLvSxdnL PFVRASGSDR
VVDSAtNWtA

            Consensus     DLTPFGEQQM VNSGIKFYRR YKAL-AR-IV PFVRASGSDR
VIASAEKFIE
                 Fcp10    DLTPFGEQQM VNSGIKFYRR YKAL.ARKIV PFVRASGSDR
VIASAEKFIE
```

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                         151
200
   A. terreus 9a1         GFQTARqDDh hAnphQPSPr VDVaIPEGsA YNNTLEHSLC
TAFEs...St
   A. terreus cbs         GFQNARqGDP hAnphQPSPr VDVVIPEGtA YNNTLEHSIC
TAFEa...St
```

```
   A. niger var. awamori GFQSTKLkDP rAqpgQSSPk IDVVISEAsS sNNTLDpGtC
TvFEd...SE
   A. niger NRRL3135     GFQSTKLkDP rAqpgQSSPk IDVVISEAsS sNNTLDpGtC
TvFEd...SE
   A. fumigatus 13073    GFQqAKLADP gAt.nRAAPa ISVIIPESeT FNNTLDHGVC
TkFEa...SQ
   A. fumigatus 32722    GFQqAKLADP gAt.nRAAPa ISVIIPESeT FNNTLDHGVC
TkFEa...SQ
   A. fumigatus 58128    GFQqAKLADP gAt.nRAAPa ISVIIPESeT FNNTLDHGVC
TkFEa...SQ
   A. fumigatus 26906    GFQqAKLADP gAt.nRAAPa ISVIIPESeT FNNTLDHGVC
TkFEa...SQ
   A. fumigatus 32239    GFQqANVADP gAt.nRAAPV ISVIIPESeT YNNTLDHSVC
TnFEa...SE
   E. nidulans           GFRkAQLhDh g.s.gQATPV VNVIIPEidG FNNTLDHStC
vSFEn...dE
   T. thermophilus       GFQSAKVlDP hSdkhDAPPt INVIIeEGpS YNNTLDtGsC
PvFEd...Ss
   T. lanuginosa         GFQdAKdrDP rSnkdQAePV INVIISEEtG sNNTLDgltC
PAaEe...Ap
   M. thermophila        GFHSAlLADR gStvrPTlPy dmVVIPETaG aNNTLHNDLC
TAFEegPySt
   Basidio               GFaxA..... ..sxntxxPx LxVILSExg. .NDTLDDNMC
......PxAG

            Consensus    GFQSAKLADP -A---QASPV INVIIPEG-G YNNTLDHGLC
TAFE--P-SE
                 Fcp10   GFQSAKLADP GANPHQASPV INVIIPEGAG YNNTLDHGLC
TAFEE...SE

                         201
250
   A. terreus 9a1        VGDDavANFT AVFAPAIaqR LEAdLPGVQL StDDVVNLMA
MCPFETVSlT
   A. terreus cbs        VGDAaADNFT AVFAPAIakR LEAdLPGVQL SADDVVNLMA
MCPFETVSlT
   A. niger var. awamori LADtVEANFT AtFAPSIRqR LEndLSGVtL TDtEVtyLMD
MCSFDTIStS
   A. niger NRRL3135     LADtVEANFT AtFvPSIRqR LEndLSGVtL TDtEVtyLMD
MCSFDTIStS
   A. fumigatus 13073    LGDEVAANFT ALFAPdIRAR aEkhLPGVtL TDEDVVSLMD
MCSFDTVArT
   A. fumigatus 32722    LGDEVAANFT ALFAPdIRAR aEkhLPGVtL TDEDVVSLMD
MCSFDTVArT
   A. fumigatus 58128    LGDEVAANFT ALFAPdIRAR aEkhLPGVtL TDEDVVSLMD
MCSFDTVArT
   A. fumigatus 26906    LGDEVAANFT ALFAPdIRAR aKkhLPGVtL TDEDVVSLMD
MCSFDTVArT
   A. fumigatus 32239    LGDEVEANFT ALFAPAIRAR IEkhLPGVQL TDDDVVSLMD
MCSFDTVArT
   E. nidulans           rADEIEANFT AIMGPPIRkR LEndLPGIKL TNENVIyLMD
MCSFDTMArT
   T. thermophilus       gGHDaQEKFA kqFAPAIlEK IKDhLPGVDL AvsDVpyLMD
LCPFETLArn
   T. lanuginosa         .DptqpAEFl qVFGPRVlkK ItkhMPGVNL TlEDVplFMD
LCPFDTVGsd
   M. thermophila        IGDDaQDtYl StFAGPItAR VNAnLPGaNL TDADtVaLMD
LCPFETVAsS
   Basidio               dSDpqxnxWl AVFAPPItAR LNAaaPGaNL TDxDaxNLxx
LCPFETVS..
```

```
                Consensus  LGDDVEANFT AVFAPPIRAR LEA-LPGVNL TDEDVVNLMD
MCPFDTVA-T
                     Fcp10 LGDDVEANFT AVFAPPIRAR LEAHLPGVNL TDEDVVNLMD
MCPFDTVART

                           251
300
   A. terreus 9a1          dD..Aht... ......LSPF CDLFTa..tE WtQYNYLlSL
dKYYGYGGGN
   A. terreus cbs          dD..Aht... ......LSPF CDLFTa..aE WtQYNYLlSL
dKYYGYGGGN
   A. niger var. awamori   Tv..DTK... ......LSPF CDLFTH..dE WiHYDYLQSL
kKYYGHGAGN
   A. niger NRRL3135       Tv..DTK... ......LSPF CDLFTH..dE WiNYDYLQSL
kKYYGHGAGN
   A. fumigatus 13073      SD..ASQ... ......LSPF CQLFTH..nE WkKYNYLQSL
gKYYGYGAGN
   A. fumigatus 32722      SD..ASQ... ......LSPF CQLFTH..nE WkKYNYLQSL
gKYYGYGAGN
   A. fumigatus 58128      SD..ASQ... ......LSPF CQLFTH..nE WkKYNYLQSL
gKYYGYGAGN
   A. fumigatus 26906      SD..ASQ... ......LSPF CQLFTH..nE WkKYNYLQSL
gKYYGYGAGN
   A. fumigatus 32239      AD..ASE... ......LSPF CAIFTH..nE WkKYDYLQSL
gKYYGYGAGN
   E. nidulans             AH..GTE... ......LSPF CAIFTE..kE WlQYDYLQSL
sKYYGYGAGS
   T. thermophilus         ht..DT.... ......LSPF CALsTQ..eE WqaYDYYQSL
gKYYGnGGGN
   T. lanuginosa           PvlfPrQ... ......LSPF CHLFTa..dD WmaYDYYyTL
dKYYSHGGGS
   M. thermophila          SsdpATadag ggngrpLSPF CrLFSE..sE WraYDYLQSV
gKWYGYGPGN
   Basidio                 .......... ...xexxSxF CDLFexxpeE FxaFxYxgdL
dKFYGtGyGQ

                Consensus  SD--ATQ--- ------LSPF CDLFTH---E W-QYDYLQSL -
KYYGYGAGN
                     Fcp10 SD..ATQ... ......LSPF CDLFTH..DE WIQYDYLQSL
GKYYGYGAGN

                           301
350
   A. terreus 9a1          PLGPvQGVGW aNELMARLTR A.PVHDHTCv NNTLDASPAT
FPLNATLYAD
   A. terreus cbs          PLGPvQGVGW aNELIARLTR S.PVHDHTCv NNTLDANPAT
FPLNATLYAD
   A. niger var. awamori   PLGPTQGVGY aNELIARLTH S.PVHDDTSS NHTLDSNPAT
FPLNSTLYAD
   A. niger NRRL3135       PLGPTQGVGY aNELIARLTH S.PVHDDTSS NHTLDSSPAT
FPLNSTLYAD
   A. fumigatus 13073      PLGPAQGIGF tNELIARLTR S.PVQDHTST NsTLvSNPAT
FPLNATMYvD
   A. fumigatus 32722      PLGPAQGIGF tNELIARLTR S.PVQDHTST NsTLvSNPAT
FPLNATMYvD
   A. fumigatus 58128      PLGPAQGIGF tNELIARLTR S.PVQDHTST NsTLvSNPAT
FPLNATMYvD
   A. fumigatus 26906      PLGPAQGIGF tNELIARLTR S.PVQDHTST NsTLvSNPAT
FPLNATMYvD
   A. fumigatus 32239      PLGPAQGIGF tNELIARLTN S.PVQDHTST NsTLDSDPAT
FPLNATIYvD
```

```
   E. nidulans          PLGPAQGIGF tNELIARLTQ S.PVQDNTST NHTLDSNPAT
FPLDrkLYAD
   T. thermophilus      PLGPAQGVGF vNELIARMTH S.PVQDYTTv NHTLDSNPAT
FPLNATLYAD
   T. lanuginosa        AFGPSRGVGF vNELIARMTg NlPVKDHTTv NHTLDdNPET
FPLDAvLYAD
   M. thermophila       PLGPTQGVGF vNELLARLA. GvPVRDgTST NRTLDGDPrT
FPLGrPLYAD
   Basidio              PLGPvQGVGY iNELLARLTx qa.VRDNTqT NRTLDSSPxT
FPLNrTFYAD

             Consensus  PLGPAQGVGF -NELIARLTH S-PVQDHTST NHTLDSNPAT
FPLNATLYAD
                  Fcp10 PLGPAQGVGF VNELIARLTH S.PVQDHTST NHTLDSNPAT
FPLNATLYAD

                        351
400
   A. terreus 9a1       FSHDSnLVSI FWALGLYNGT aPLSqTSVE. .SvsQTDGYA
AAWTVPFAAR
   A. terreus cbs       FSHDSnLVSI FWALGLYNGT kPLSqTTVE. .ditrTDGYA
AAWTVPFAAR
   A. niger var. awamori FSHDNGIISI LFALGLYNGT kPLSTTTVE. .NitQTDGFS
SAWTVPFASR
   A. niger NRRL3135    FSHDNGIISI LFALGLYNGT kPLSTTTVE. .NitQTDGFS
SAWTVPFASR
   A. fumigatus 13073   FSHDNSMVSI FFALGLYNGT ePLSrTSVE. .SaKElDGYS
ASWvVPFGAR
   A. fumigatus 32722   FSHDNSMVSI FFALGLYNGT gPLSrTSVE. .SaKElDGYS
ASWvVPFGAR
   A. fumigatus 58128   FSHDNSMVSI FFALGLYNGT ePLSrTSVE. .SaKElDGYS
ASWvVPFGAR
   A. fumigatus 26906   FSHDNSMVSI FFALGLYNGT ePLSrTSVE. .SaKElDGYS
ASWvVPFGAR
   A. fumigatus 32239   FSHDNGMIPI FFAMGLYNGT ePLSqTSeE. .StKESNGYS
ASWAVPFGAR
   E. nidulans          FSHDNSMISI FFAMGLYNGT qPLSmdSVE. .SiQEmDGYA
ASWTVPFGAR
   T. thermophilus      FSHDNTMtSI FaALGLYNGT akLSTTeIK. .SiEETDGYS
AAWTVPFGGR
   T. lanuginosa        FSHDNTMtGI FsAMGLYNGT kPLSTSkIQP pTgAAADGYA
ASWTVPFAAR
   M. thermophila       FSHDNdMMGV LgALGaYDGv pPLdkTA..R rdpEElGGYA
ASWAVPFAAR
   Basidio              FSHDNqMVAI FsAMGLFNqS aPLdPSxpDP nrt....Wv
TSklVPFSAR

             Consensus  FSHDNTMVSI FFALGLYNGT -PLSTTSVEP -S-EETDGYA
ASWTVPFAAR
                  Fcp10 FSHDNTMVSI FFALGLYNGT KPLSTTSVE. .SIEETDGYA
ASWTVPFAAR

                        401
450
   A. terreus 9a1       AYVEMMQC.. ra..... .....EKEPL VRVLVNDRVM
PLHGCPtDKL
   A. terreus cbs       AYIEMMQC.. ra..... .....EKQPL VRVLVNDRVM
PLHGCAVDNL
   A. niger var. awamori lYVEMMQC.. Qa..... .....EQEPL VRVLVNDRVV
PLHGCPIDaL
   A. niger NRRL3135    lYVEMMQC.. Qa..... .....EQEPL VRVLVNDRVV
PLHGCPVDaL
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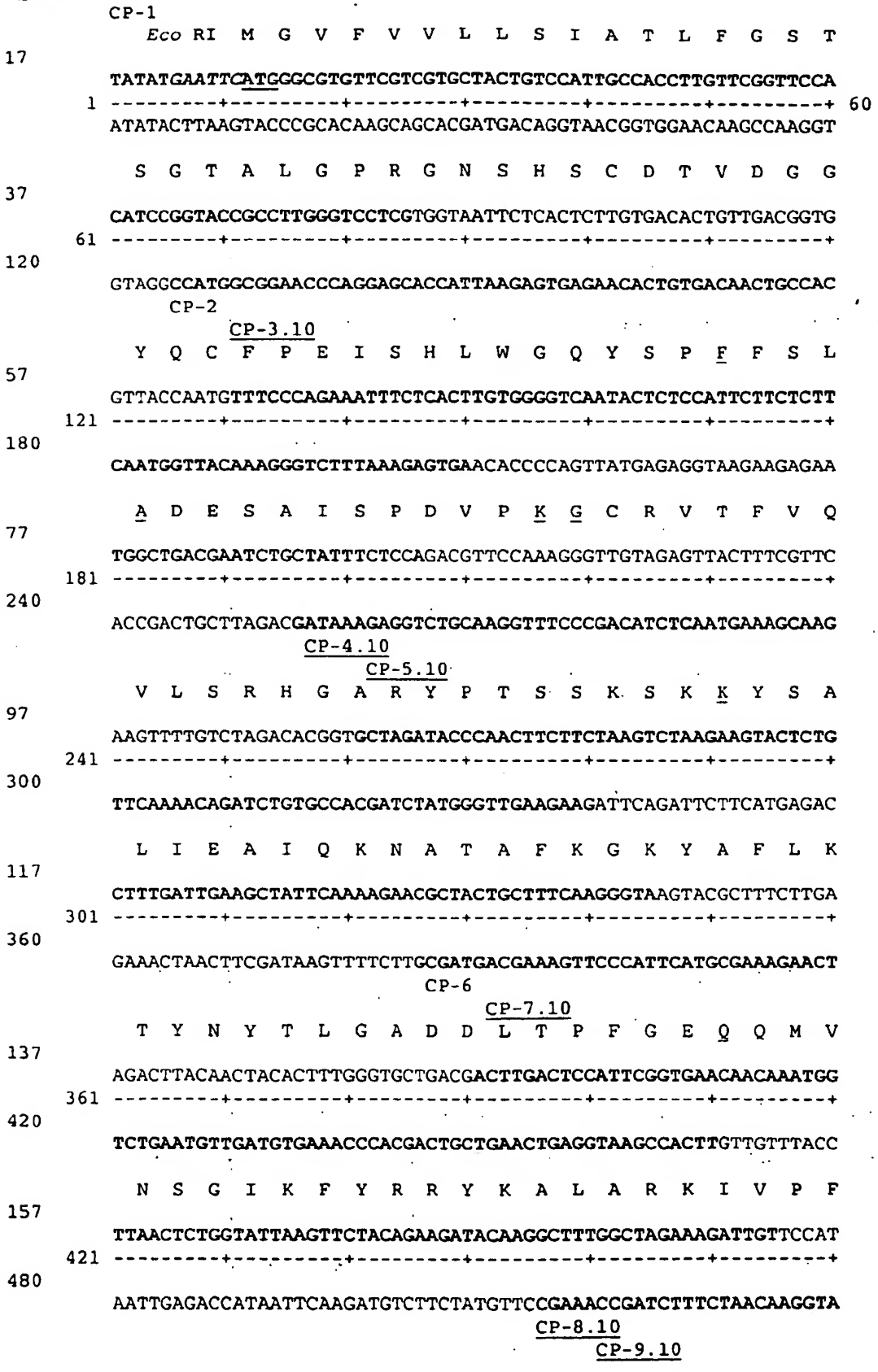
   A. fumigatus 13073    AYfEtMQC.. Ks........ .....EKEPL VRaLINDRVV
PLHGCDVDKL
   A. fumigatus 32722    AYfEtMQC.. Ks........ .....EKEPL VRaLINDRVV
PLHGCDVDKL
   A. fumigatus 58128    AYfEtMQC.. Ks........ .....EKESL VRaLINDRVV
PLHGCDVDKL
   A. fumigatus 26906    AYfEtMQC.. Ks........ .....EKEPL VRaLINDRVV
PLHGCDVDKL
   A. fumigatus 32239    AYfEtMQC.. Ks........ .....EKEPL VRaLINDRVV
PLHGCAVDKL
   E. nidulans           AYfELMQC.. E......... .....KKEPL VRVLVNDRVV
PLHGCAVDKF
   T. thermophilus       AYIEMMQC.. Dd........ .....sDEPV VRVLVNDRVV
PLHGCEVDsL
   T. lanuginosa         AYVELLRC.. Etetsseeee EG...EDEPF VRVLVNDRVV
PLHGCrVDRW
   M. thermophila        iYVEkMRC.. sggggggggg EGrqeKDEeM VRVLVNDRVM
TLkGCGaDEr
   Basidio               mvVErLxCxx xgtxxxxxxx xxxxxxxxxx VRVLVNDaVq
PLEfCGgDxd

             Consensus   AYVEMMQC-- E--------- EG---EKEPL VRVLVNDRVV
PLHGCGVDKL
                 Fcp10   AYVEMMQC.. EA........ .....EKEPL VRVLVNDRVV
PLHGCGVDKL
```

	451			482
A. terreus 9a1	GRCKrDAFVA	GLSFAQAG..	GNWADCF~~~	~~
A. terreus cbs	GRCKrDDFVE	GLSFARAG..	GNWAECF~~~	~~
A. niger var. *awamori*	GRCtrDsFVr	GLSFARSG..	GDWAECsA~~	~~
A. niger NRRL3135	GRCtrDsFVr	GLSFARSG..	GDWAECFA~~	~~
A. fumigatus 13073	GRCKlNDFVK	GLSWARSG..	GNWGECFS~~	~~
A. fumigatus 32722	GRCKlNDFVK	GLSWARSG..	GNWGECFS~~	~~
A. fumigatus 58128	GRCKlNDFVK	GLSWARSG..	GNWGECFS~~	~~
A. fumigatus 26906	GRCKlNDFVK	GLSWARSG..	GNWGECFS~~	~~
A. fumigatus 32239	GRCKlKDFVK	GLSWARSG..	GNSEQSFS~~	~~
E. nidulans	GRCtlDDWVE	GLNFARSG..	GNWKtCFTl~	~~
T. thermophilus	GRCKrDDFVr	GLSFARqG..	GNWEGCYAas	e~
T. lanuginosa	GRCRrDEWIK	GLTFARqG..	GHWDrCF~~~	~~
M. thermophila	GmCtlErFIE	SMAFARGN..	GKWDlCFA~~	~~
Basidio	GxCtlDAFVE	SqxYAReDgq	GDFEKCFAtp	xx
Consensus	GRCK-DDFVE	GLSFARSG--	GNWEECFA--	--
Fcp10	**GRCKRDDFVE**	**GLSFARSG..**	**GNWEECFA..**	**..**

Figure 17



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          V  R  A  S  G  S  D  R  V  I  A  S  A  E  K  F  I  E  G  F
177
      TCGTTAGAGCTTCTGGTTCTGACAGAGTTATTGCTTCTGCTGAAAAGTTCATTGAAGGTT
  481 ---------+---------+---------+---------+---------+---------+
  540
      AGCAATCTCGAAGACCAAGACTGTCTCAATAACGAAGACGACTTTTCAAGTAACTTCCAA

          Q  S  A  K  L  A  D  P  G  A  N  P  H  Q  A  S  P  V  I  N
  197
      TCCAATCTGCTAAGTTGGCTGACCCAGGTGCTAACCCACACCAAGCTTCTCCAGTTATTA
  541 ---------+---------+---------+---------+---------+---------+
  600
      AGGTTAGACGATTCAACCGACTGGGTCCACGATTGGGTGTGGTTCGAAGAGGTCAATAAT
                                                    CP-10.10
                                                       CP-11.10
          V  I  I  P  E  G  A  G  Y  N  N  T  L  D  H  G  L  C  T  A
  217
      ACGTTATTATTCCAGAAGGTGCTGGTTACAACAACACTTTGGACCACGGTTTGTGTACTG
  601 ---------+---------+---------+---------+---------+---------+
  660
      TGCAATAATAAGGTCTTCCACGACCAATGTTGTTGTGAAACCTGGTGCCAAACACATGAC

          F  E  E  S  E  L  G  D  D  V  E  A  N  F  T  A  V  F  A  P
  237
      CTTTCGAAGAATCTGAATTGGGTGACGACGTTGAAGCTAACTTCACTGCTGTTTTCGCTC
  661 ---------+---------+---------+---------+---------+---------+
  720
      GAAAGCTTCTTAGACTTAACCCACTGCTGCAACTTCGATTGAAGTGACGACAAAAGCGAG
                                                                CP-12.10

          P  I  R  A  R  L  E  A  H  L  P  G  V  N  L  T  D  E  D  V
  257
      CACCTATTAGAGCTAGATTGGAAGCTCACTTGCCAGGTGTTAACTTGACTGACGAAGACG
  721 ---------+---------+---------+---------+---------+---------+
  780
      GTGGATAATCTCGATCTAACCTTCGAGTGAACGGTCCACAATTGAACTGACTGCTTCTGC

      CP-13.10
          V  N  L  M  D  M  C  P  F  D  T  V  A  R  T  S  D  A  T  Q
  277
      TTGTTAACTTGATGGACATGTGTCCATTCGACACTGTTGCTAGAACTTCTGACGCTACTC
  781 ---------+---------+---------+---------+---------+---------+
  840
      AACAATTGAACTACCTGTACACAGGTAAGCTGTGACAACGATCTTGAAGACTGCGATGAG

          L  S  P  F  C  D  L  F  T  H  D  E  W  I  Q  Y  D  Y  L  Q
  297
      AATTGTCTCCATTCTGTGACTTGTTCACTCACGACGAATGGATTCAATACGACTACTTGC
  841 ---------+---------+---------+---------+---------+---------+
  900
      TTAACAGAGGTAAGACACTGAACAAGTGAGTGCTGCTTACCTAAGTTATGCTGATGAACG
            CP-14.10
               CP-15.10
          S  L  G  K  Y  Y  G  Y  G  A  G  N  P  L  G  P  A  Q  G  V
  317
      AATCTTTGGGTAAGTACTACGGTTACGGTGCTGGTAACCCATTGGGTCCAGCTCAAGGTG
  901 ---------+---------+---------+---------+---------+---------+
  960
      TTAGAAACCCATTCATGATGCCAATGCCACGACCATTGGGTAACCCAGGTCGAGTTCCAC

          G  F  V  N  E  L  I  A  R  L  T  H  S  P  V  Q  D  H  T  S
  337
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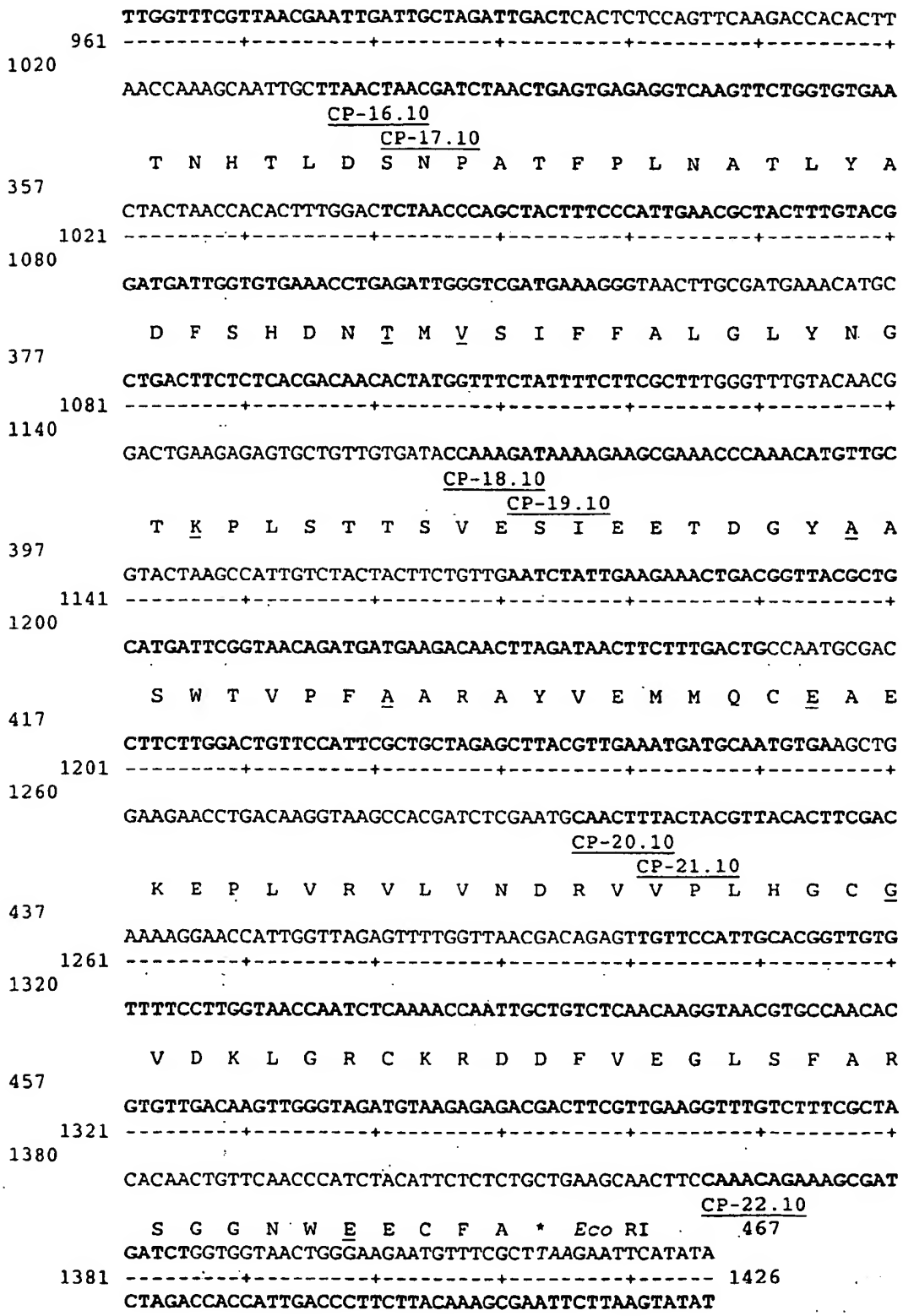


Figure 18

```
                                1
50
P. involutus (phyA1)            ---------- ~FPipeseqR nWSPYSPYFP LAEykA....
pPaGCQInqV
P. involutus (phyA2)            ---------- ~FsipeseqR nWSPYSPYFP LAEykA....
pPaGCeInqV
T. pubescens                    ---------- ~LDvtRDVqQ sWSmYSPYFP aAtyvA....
pPaSCQInqV
A. pediades                     ---------- ~pffpPQIqD sWAaYTPYYP VqAyTP....
pPKDCKITqV
P. lycii                        ---------- ~LPipAQnTs nWGPYdPFFP VEpyAA....
pPEGCtVTqV
A. terreus 9a1                  KhsdCNSVDh GYQCfPELSH kWGlYAPYFS LqDESPFPlD
VPEDCHITFV
A. terreus cbs                  NhsdCtSVDr GYQCfPELSH kWGlYAPYFS LqDESPFPlD
VPDDCHITFV
A. niger var. awamori           NqsTCDTVDq GYQCfSEtSH LWGQYAPFFS LANESAISPD
VPaGCRVTFa
A. niger T213                   NqsSCDTVDq GYQCfSEtSH LWGQYAPFFS LANESvISPD
VPaGCRVTFa
A. niger NRRL3135               NqsSCDTVDq GYQCfSEtSH LWGQYAPFFS LANESvISPE
VPaGCRVTFa
A. fumigatus ATCC13073          GSkSCDTVDl GYQCsPAtSH LWGQYSPFFS LEDElSVSSK
LPKDCRITLV
A. fumigatus ATCC32722          GSkSCDTVDl GYQCsPAtSH LWGQYSPFFS LEDElSVSSK
LPKDCRITLV
A. fumigatus ATCC58128          GSkSCDTVDl GYQCsPAtSH LWGQYSPFFS LEDElSVSSK
LPKDCRITLV
A. fumigatus ATCC26906          GSkSCDTVDl GYQCsPAtSH LWGQYSPFFS LEDElSVSSK
LPKDCRITLV
A. fumigatus ATCC32239          GSkACDTVEl GYQCsPGtSH LWGQYSPFFS LEDElSVSSD
LPKDCRVTFV
E. nidulans                     QNHSCNTaDg GYQCfPNVSH VWGQYSPYFS IEQESAISeD
VPhGCeVTFV
T. thermophilus                 DSHSCNTVEg GYQCrPEISH sWGQYSPFFS LADQSEISPD
VPQNCKITFV
T. lanuginosa                   ---------- ----nvDIAR hWGQYSPFFS LAEvSEISPA
VPKGCRVeFV
M. thermophila                  ESRPCDTpDl GFQCgTAISH FWGQYSPYFS VPsElDaS..
IPDDCeVTFa

Consensus Seq. 11               NSHSCDTVD- GYQC-PEISH LWGQYSPFFS LADESAISPD
VPKGCRVTFV

                                51
100
P. involutus (phyA1)            NIIqRHGARF PTSGaTtRik AgLtKLQgvq nftDAKFnFI
KSFKYdLGns
P. involutus (phyA2)            NIIqRHGARF PTSGaAtRik AgLsKLQsvq nftDPKFDFI
KSFtYdLGTs
T. pubescens                    HIIqRHGARF PTSGaAKRiq TaVAKLKaaS nytDPlLAFV
tnYtYSLGqD
A. pediades                     NIIqRHGARF PTSGaGtRiq AaVKKLQsak TytDPRLDFL
tnYtYTLGhD
P. lycii                        NLIqRHGARW PTSGarsRqv AaVAKIQmar PftDPKYEFL
NdFvYkFGvA
A. terreus 9a1                  QVLARHGARs PThSKTKaYA AtIAaIQKSA TaFpGKYAFL
QSYNYSLDSE
A. terreus cbs                  QVLARHGARs PTdSKTKaYA AtIAaIQKNA TaLpGKYAFL
KSYNYSMGSE
```

A. niger var. *awamori* QVLSRHGARY PTeSKGKKYS ALIEeIQQNv TtFDGKYAFL
KTYNYSLGAD
A. niger T213 QVLSRHGARY PTeSKGKKYS ALIEeIQQNv TtFDGKYAFL
KTYNYSLGAD
A. niger NRRL3135 QVLSRHGARY PTdSKGKKYS ALIEeIQQNA TtFDGKYAFL
KTYNYSLGAD
A. fumigatus ATCC13073 QVLSRHGARY PTSSKSKKYk kLVtaIQaNA TdFKGKFAFL
KTYNYTLGAD
A. fumigatus ATCC32722 QVLSRHGARY PTSSKSKKYk kLVtaIQaNA TdFKGKFAFL
KTYNYTLGAD
A. fumigatus ATCC58128 QVLSRHGARY PTSSKSKKYk kLVtaIQaNA TdFKGKFAFL
KTYNYTLGAD
A. fumigatus ATCC26906 QVLSRHGARY PTSSKSKKYk kLVtaIQaNA TdFKGKFAFL
KTYNYTLGAD
A. fumigatus ATCC32239 QVLSRHGARY PTASKSKKYk kLVtaIQKNA TeFKGKFAFL
ETYNYTLGAD
E. nidulans QVLSRHGARY PTeSKSKaYS GLIEaIQKNA TsFwGQYAFL
ESYNYTLGAD
T. thermophilus QLLSRHGARY PTSSKTElYS qLIsRIQKtA TaYKGyYAFL
KdYrYqLGAN
T. lanuginosa QVLSRHGARY PTAhKSEvYA ELLQRIQDtA TeFKGDFAFL
RdYaYhLGAD
M. thermophila QVLSRHGARa PTlkRAasYv DLIDRIHhGA isYgPgYEFL
RTYDYTLGAD

Consensus Seq. 11 **QVLSRHGARY PTSSKSKKYS ALIERIQKNA T-FKGKYAFL**
KTYNYTLGAD

101
150
P. involutus (phyA1) DLvPFGAaQs fDAGqEaFaR YskLvSKNnL PFIRAdGSDR
VVDSAtNWtA
P. involutus (phyA2) DLvPFGAaQs fDAGLEvFaR YskLvSsDnL PFIRSdGSDR
VVDTAtNWtA
T. pubescens sLveLGAtQs sEAGqEaFtR YsSLvSaDeL PFVRASGSDR
VVATANNWtA
A. pediades DLvPFGAlQs sQAGeEtFQR YsfLvSKEnL PFVRASSSNR
VVDSAtNWtE
P. lycii DLlPFGANQs hQTGtDMYtR YsTLfEgGdV PFVRAAGdQR
VVDSStNWtA
A. terreus 9a1 ELTPFGrNQL rDlGaQFYeR YNAL.TRHIn PFVRATDAsR
VhESAEKFVE
A. terreus cbs NLTPFGrNQL qDlGaQFYRR YDTL.TRHIn PFVRAADSsR
VhESAEKFVE
A. niger var. *awamori* DLTPFGEQEL VNSGIKFYQR YESL.TRNII PFIRSSGSsR
VIASGEKFIE
A. niger T213 DLTPFGEQEL VNSGIKFYQR YESL.TRNII PFIRSSGSsR
VIASGEKFIE
A. niger NRRL3135 DLTPFGEQEL VNSGIKFYQR YESL.TRNIV PFIRSSGSsR
VIASGKKFIE
A. fumigatus ATCC13073 DLTPFGEQQL VNSGIKFYQR YKAL.ARSVV PFIRASGSDR
VIASGEKFIE
A. fumigatus ATCC32722 DLTPFGEQQL VNSGIKFYQR YKAL.ARSVV PFIRASGSDR
VIASGEKFIE
A. fumigatus ATCC58128 DLTPFGEQQL VNSGIKFYQR YKAL.ARSVV PFIRASGSDR
VIASGEKFIE
A. fumigatus ATCC26906 DLTAFGEQQL VNSGIKFYQR YKAL.ARSVV PFIRASGSDR
VIASGEKFIE
A. fumigatus ATCC32239 DLTPFGEQQM VNSGIKFYQK YKAL.AgSVV PFIRSSGSDR
VIASGEKFIE
E. nidulans DLTiFGENQM VDSGaKFYRR YKnL.ARKnt PFIRASGSDR
VVASAEKFIN

T. thermophilus VIASGrlFIE	DLTPFGENQM IQlGIKFYnH YKSL.ARNaV	PFVRCSGSDR
T. lanuginosa VIASAEfFnr	NLTRFGEEQM MESGrQFYHR YREq.AREIV	PFVRAAGSAR
M. thermophila VVhSAENFtQ	ELTRtGQQQM VNSGIKFYRR YRAL.ARKsI	PFVRTAGqDR
Consensus Seq. 11 VIASAEKFIE	**DLTPFGENQM VNSGIKFYRR YKAL-ARNIV**	**PFVRASGSDR**

151

200

P. involutus (phyA1) PAaGD.....	GFaSA..... ..shNtvqPk LNLILPQ..T	gNDTLEDNMC
P. involutus (phyA2) PAaGE.....	GFaSA..... ..srNaiqPk LDLILPQ..T	gNDTLEDNMC
T. pubescens PAaGD.....	GFalA..... ..ssNsiTPV LSVIISE..A	gNDTLDDNMC
A. pediades PnaGs.....	GFsAA..... ..shHvlNPI LfVILSE..S	LNDTLDDAMC
P. lycii PnevD.....	GFgdA..... ..sgEtvlPt LQVVLQE..E	gNcTLcNNMC
A. terreus 9a1 TAFEs...ST	GFQTARqDDh hAnpHQPSPr VDVaIPEGSA	YNNTLEHSLC
A. terreus cbs TAFEA...ST	GFQNARqGDP hAnpHQPSPr VDVVIPEGTA	YNNTLEHSIC
A. niger var. *awamori* TvFED...Se	GFQSTKLkDP rAqpgQSSPk IDVVISEASS	sNNTLDpGtC
A. niger T213 TvFED...Se	GFQSTKLkDP rAqpgQSSPk IDVVISEASS	sNNTLDpGtC
A. niger NRRL3135 TvFED...Se	GFQSTKLkDP rAqpgQSSPk IDVVISEASS	sNNTLDpGtC
A. fumigatus ATCC13073 TkFEA...Sq	GFQqAKLADP gAt.NRAAPa ISVIIPESeT	FNNTLDHGVC
A. fumigatus ATCC32722 TkFEA...Sq	GFQqAKLADP gAt.NRAAPa ISVIIPESeT	FNNTLDHGVC
A. fumigatus ATCC58128 TkFEA...Sq	GFQqAKLADP gAt.NRAAPa ISVIIPESeT	FNNTLDHGVC
A. fumigatus ATCC26906 TkFEA...Sq	GFQqAKLADP gAt.NRAAPa ISVIIPESeT	FNNTLDHGVC
A. fumigatus ATCC32239 TnFEA...Se	GFQqANVADP gAt.NRAAPV ISVIIPESeT	YNNTLDHSVC
E. nidulans vSFEN...de	GFRkAQLhDh g.s.gQATPV VNVIIPEidG	FNNTLDHStC
T. thermophilus PvFED...SS	GFQSAKVlDP hSdkHDAPPt INVIIeEGPS	YNNTLDtGsC
T. lanuginosa PAaEE...AP	GFQdAKdrDP rSnkDQAePV INVIISEETG	sNNTLDgltC
M. thermophila TAFEEgpyST	GFHSAlLADR gStvRPTlPy dmVVIPETAG	aNNTLHNDLC
Consensus Seq. 11 TAFED---ST	**GFQSAKLADP -A--HQASPV INVIIPEGSG**	**YNNTLDHGLC**

201

250

P. involutus (phyA1) LCAFlTVSK.	.SDpqvnaWl AVafPSItAR LNAaaPSVNL	TDtDafNLVs
P. involutus (phyA2) LCPFmTVSK.	.SDpqvDaWl AsafPSVtAQ LNAaaPGaNL	TDADafNLVs
T. pubescens LCPFETVAt.	.SDpqvnQWl AqFAPPMtAR LNAgaPGaNL	TDtDtyNLLt

A. pediades	.SDpqtGiWT	SIYGTPIanR	LNqqaPGaNI	TAADVsNLIp
LCAFETIvK.				
P. lycii	.GDESt.tWl	GVFAPnItAR	LNAaaPSaNL	SDsDaLtLMD
MCPFDTLSs.				
A. terreus 9a1	VGDDAvANFT	AVFAPAIaqR	LEAdLPGVQL	StDDVVNLMA
MCPFETVSlT				
A. terreus cbs	VGDAAADNFT	AVFAPAIakR	LEAdLPGVQL	SADDVVNLMA
MCPFETVSlT				
A. niger var. *awamori*	LADtvEANFT	AtFAPSIRqR	LEndLSGVtL	TDtEVtyLMD
MCSFDTIStS				
A. niger T213	LADtvEANFT	AtFAPSIRqR	LEndLSGVtL	TDtEVtyLMD
MCSFDTIStS				
A. niger NRRL3135	LADtvEANFT	AtFvPSIRqR	LEndLSGVtL	TDtEVtyLMD
MCSFDTIStS				
A. fumigatus ATCC13073	LGDEvAANFT	ALFAPdIRAR	aEkhLPGVtL	TDEDVVSLMD
MCSFDTVART				
A. fumigatus ATCC32722	LGDEvAANFT	ALFAPdIRAR	aEkhLPGVtL	TDEDVVSLMD
MCSFDTVART				
A. fumigatus ATCC58128	LGDEvAANFT	ALFAPdIRAR	aEkhLPGVtL	TDEDVVSLMD
MCSFDTVART				
A. fumigatus ATCC26906	LGDEvAANFT	ALFAPdIRAR	aKkhLPGVtL	TDEDVVSLMD
MCSFDTVART				
A. fumigatus ATCC32239	LGDEvEANFT	ALFAPAIRAR	IEkhLPGVQL	TDDDVVSLMD
MCSFDTVART				
E. nidulans	rADEiEANFT	AIMGPPIRkR	LEndLPGIKL	TNENVIyLMD
MCSFDTMART				
T. thermophilus	gGHDAQEKFA	kqFAPAIlEK	IKDhLPGVDL	AvsDVpyLMD
LCPFETLARn				
T. lanuginosa	.DptqpAEFl	qVFGPRVlkK	ItkhMPGVNL	TlEDVplFMD
LCPFDTVGsd				
M. thermophila	IGDDAQDtYl	StFAGPItAR	VNAnLPGaNL	TDADtVaLMD
LCPFETVAsS				
Consensus Seq. 11	**LGDDAEANFT**	**AVFAPPIRAR**	**LEA-LPGVNL**	**TDEDVVNLMD**
MCPFDTVART				

251

300				
P. involutus (*phyA1*)	.....	....ekkSdF	CtLFegiPGs	FeaFAYggdL
dKFYGtGyGQ				
P. involutus (*phyA2*)	.....	....eqkSdF	CtLFegiPGs	FeaFAYagdL
dKFYGtGyGQ				
T. pubescens	.....	....errSeF	CDIYeelqAE	.daFAYnadL
dKFYGtGyGQ				
A. pediades	.....	....etpSPF	CNLF..TPEE	FaQFEYFgdL
dKFYGtGyGQ				
P. lycii	.....	....gnaSPF	CDLF..TAEE	YvsYEYYydL
dKYYGtGPGN				
A. terreus 9a1	dD..Aht...	......LSPF	CDLF..TAtE	WtQYNYLlSL
dKYYGYGGGN				
A. terreus cbs	dD..Aht...	......LSPF	CDLF..TAAE	WtQYNYLlSL
dKYYGYGGGN				
A. niger var. *awamori*	Tv..DTK...	......LSPF	CDLF..ThDE	WiHYDYLQSL
kKYYGHGAGN				
A. niger T213	Tv..DTK...	......LSPF	CDLF..ThDE	WiHYDYLRSL
kKYYGHGAGN				
A. niger NRRL3135	Tv..DTK...	......LSPF	CDLF..ThDE	WiNYDYLQSL
kKYYGHGAGN				
A. fumigatus ATCC13073	SD..ASQ...	......LSPF	CQLF..ThNE	WkKYNYLQSL
gKYYGYGAGN				
A. fumigatus ATCC32722	SD..ASQ...	......LSPF	CQLF..ThNE	WkKYNYLQSL
gKYYGYGAGN				

A. fumigatus ATCC58128	SD..ASQ... ......LSPF CQLF..ThNE WkKYNYLQSL gKYYGYGAGN	
A. fumigatus ATCC26906	SD..ASQ... ......LSPF CQLF..ThNE WkKYNYLQSL gKYYGYGAGN	
A. fumigatus ATCC32239	AD..ASE... ......LSPF CAIF..ThNE WkKYDYLQSL gKYYGYGAGN	
E. nidulans	AH..GTE... ......LSPF CAIF..TEKE WlQYDYLQSL sKYYGYGAGS	
T. thermophilus	ht..DT.... ......LSPF CALs..TqEE WqaYDYYQSL gKYYGnGGGN	
T. lanuginosa	PvlfPrQ... ......LSPF CHLF..TADD WmaYDYYyTL dKYYSHGGGS	
M. thermophila	SsdpATadag ggngrpLSPF CrLF..SEsE WraYDYLQSV gKWYGYGPGN	
Consensus Seq. 11	**SD--ATQ--- ------LSPF CDLF--TADE W-QYDYLQSL - KYYGYGAGN**	

301

350

P. involutus (phyA1)	eLGPvQGVGY vNELIARLTN S.AVRDNTqT NRTLDASPvT FPLNkTFYAD	
P. involutus (phyA2)	ALGPvQGVGY iNELLARLTN S.AVNDNTqT NRTLDAaPDT FPLNkTMYAD	
T. pubescens	PLGPvQGVGY iNELIARLTa q.nVsDHTqT NsTLDSSPET FPLNrTLYAD	
A. pediades	PLGPvQGVGY iNELLARLTE m.PVRDNTqT NRTLDSSPlT FPLDrSIYAD	
P. lycii	ALGPvQGVGY vNELLARLTg q.AVRDETqT NRTLDSDPAT FPLNrTFYAD	
A. terreus 9a1	PLGPvQGVGW aNELMARLTR A.PVHDHTCv NNTLDASPAT FPLNATLYAD	
A. terreus cbs	PLGPvQGVGW aNELIARLTR S.PVHDHTCv NNTLDANPAT FPLNATLYAD	
A. niger var. *awamori*	PLGPTQGVGY aNELIARLTH S.PVHDDTSS NHTLDSNPAT FPLNSTLYAD	
A. niger T213	PLGPTQGVGY aNELIARLTH S.PVHDDTSS NHTLDSNPAT FPLNSTLYAD	
A. niger NRRL3135	PLGPTQGVGY aNELIARLTH S.PVHDDTSS NHTLDSSPAT FPLNSTLYAD	
A. fumigatus ATCC13073	PLGPAQGIGF tNELIARLTR S.PVQDHTST NsTLvSNPAT FPLNATMYvD	
A. fumigatus ATCC32722	PLGPAQGIGF tNELIARLTR S.PVQDHTST NsTLvSNPAT FPLNATMYvD	
A. fumigatus ATCC58128	PLGPAQGIGF tNELIARLTR S.PVQDHTST NsTLvSNPAT FPLNATMYvD	
A. fumigatus ATCC26906	PLGPAQGIGF tNELIARLTR S.PVQDHTST NsTLvSNPAT FPLNATMYvD	
A. fumigatus ATCC32239	PLGPAQGIGF tNELIARLTN S.PVQDHTST NsTLDSDPAT FPLNATIYvD	
E. nidulans	PLGPAQGIGF tNELIARLTQ S.PVQDNTST NHTLDSNPAT FPLDrkLYAD	
T. thermophilus	PLGPAQGVGF vNELIARMTH S.PVQDYTTv NHTLDSNPAT FPLNATLYAD	
T. lanuginosa	AFGPSRGVGF vNELIARMTg NlPVKDHTTv NHTLDdNPET FPLDAvLYAD	
M. thermophila	PLGPTQGVGF vNELLARLA. GvPVRDgTST NRTLDGDPrT FPLGrPLYAD	
Consensus Seq. 11	**PLGPAQGVGF -NELIARLTH S-PVQDHTST NHTLDSNPAT FPLNATLYAD**	

```
                                351
400
P. involutus (phyA1)      FSHDNlMVAV FsAMGLFrqP aPLSTSvpNP wrt.....Wr
TSSlVPFSGR
P. involutus (phyA2)      FSHDNlMVAV FsAMGLFrqS aPLSTSTpDP nrt.....Wl
TSSvVPFSAR
T. pubescens              FSHDNqMVAI FsAMGLFNqS aPLdPTTpDP art.....Fl
vkkiVPFSAR
A. pediades               LSHDNqMIAI FsAMGLFNqS sPLdPSfpNP krt.....Wv
TSRltPFSAR
P. lycii                  FSHDNTMVPI FaALGLFNAT a.LdPlkpDe nrl.....Wv
DSklVPFSGH
A. terreus 9a1            FSHDSnLVSI FWALGLYNGT aPLSqTSVES Vs..QTDGYA
AAWTVPFAAR
A. terreus cbs            FSHDSnLVSI FWALGLYNGT KPLSqTTVEd It..rTDGYA
AAWTVPFAAR
A. niger var. awamori     FSHDNGIISI LFALGLYNGT KPLSTTTVEN It..QTDGFS
SAWTVPFASR
A. niger T213             FSHDNGIISI LFALGLYNGT KPLSTTTVEN It..QTDGFS
SAWTVPFASR
A. niger NRRL3135         FSHDNGIISI LFALGLYNGT KPLSTTTVEN It..QTDGFS
SAWTVPFASR
A. fumigatus ATCC13073    FSHDNSMVSI FFALGLYNGT EPLSrTSVES ak..ElDGYS
ASWvVPFGAR
A. fumigatus ATCC32722    FSHDNSMVSI FFALGLYNGT gPLSrTSVES ak..ElDGYS
ASWvVPFGAR
A. fumigatus ATCC58128    FSHDNSMVSI FFALGLYNGT EPLSrTSVES ak..ElDGYS
ASWvVPFGAR
A. fumigatus ATCC26906    FSHDNSMVSI FFALGLYNGT EPLSrTSVES ak..ElDGYS
ASWvVPFGAR
A. fumigatus ATCC32239    FSHDNGMIPI FFAMGLYNGT EPLSqTSeES tk..ESNGYS
ASWAVPFGAR
E. nidulans               FSHDNSMISI FFAMGLYNGT QPLSmdSVES Iq..EmDGYA
ASWTVPFGAR
T. thermophilus           FSHDNTMtSI FaALGLYNGT akLSTTeIKS Ie..ETDGYS
AAWTVPFGGR
T. lanuginosa             FSHDNTMtGI FsAMGLYNGT KPLSTSkIQP ptgaAADGYA
ASWTVPFAAR
M. thermophila            FSHDNdMMGV LgALGaYDGv pPLdkTArrd ..peElGGYA
ASWAVPFAAR

Consensus Seq. 11         FSHDNTMVSI FFALGLYNGT KPLSTTSVES I---ETDGYA
ASWTVPFAAR

                                401
450
P. involutus (phyA1)      mvVErLsC.. fGt..... ........Tk VRVLVQDQVq
PLEfCGgDRn
P. involutus (phyA2)      maVErLsC.. AGt..... .......Tk VRVLVQDQVq
PLEfCGgDQd
T. pubescens              mvVErLDC.. GGa..... ........Qs VRLLVNDaVq
PLafCGaDts
A. pediades               mvtErLlCQr DGtGsGGpsr imrNgnvQTF VRILVNDaLq
PLkfCGgDmd
P. lycii                  mtVEkLaC.. ..... .....sgKea VRVLVNDaVq
PLEfCGg.vd
A. terreus 9a1            AYVEMMQCrA ..... ..EK...EPL VRVLVNDRVM
PLHGCPtDKL
A. terreus cbs            AYIEMMQCrA ..... ..EK...QPL VRVLVNDRVM
PLHGCAVDNL
A. niger var. awamori     lYVEMMQCQA ..... ..EQ...EPL VRVLVNDRVV
PLHGCPIDaL
```

A. niger T213	lYVEMMQCQA	..EQ...EPL VRVLVNDRVV PLHGCPIDaL
A. niger NRRL3135	lYVEMMQCQA	..EQ...EPL VRVLVNDRVV PLHGCPVDaL
A. fumigatus ATCC13073	AYfEtMQCKS	..EK...EPL VRaLINDRVV PLHGCDVDKL
A. fumigatus ATCC32722	AYfEtMQCKS	..EK...EPL VRaLINDRVV PLHGCDVDKL
A. fumigatus ATCC58128	AYfEtMQCKS	..EK...ESL VRaLINDRVV PLHGCDVDKL
A. fumigatus ATCC26906	AYfEtMQCKS	..EK...EPL VRaLINDRVV PLHGCDVDKL
A. fumigatus ATCC32239	AYfEtMQCKS	..EK...EPL VRaLINDRVV PLHGCAVDKL
E. nidulans	AYfELMQCE.	..KK...EPL VRVLVNDRVV PLHGCAVDKF
T. thermophilus	AYIEMMQCDD	..sD...EPV VRVLVNDRVV PLHGCEVDsL
T. lanuginosa	AYVELLRCET ETsSeEEeEG	..ED...EPF VRVLVNDRVV PLHGCrVDRW
M. thermophila	iYVEkMRCsG GGgGgGGgEG	..rQekdEeM VRVLVNDRVM TLkGCGaDEr
Consensus Seq. 11	**AYVEMMQCEA GG-G-GG-EG**	**--EK---EPL VRVLVNDRVV PLHGCGVDKL**

	451	482
P. involutus (phyA1)	GlCtLAKFVE SqTFARSDga GDFEKCFAts	a~
P. involutus (phyA2)	GlCaLDKFVE SqAYARSGga GDFEKCLAtt	v~
T. pubescens	GvCtLDAFVE SqAYARNDge GDFEKCFAt~	~~
A. pediades	SlCtLEAFVE SqkYAReDgq GDFEKCFD~~	~~
P. lycii	GvCELsAFVE SqTYAReNgq GDFAKCgfvp	se
A. terreus 9a1	GRCKrDAFVA GLSFAQAG.. GNWADCF~~~	~~
A. terreus cbs	GRCKrDDFVE GLSFARAG.. GNWAECF~~~	~~
A. niger var. *awamori*	GRCtrDsFVr GLSFARSG.. GDWAECsA~~	~~
A. niger T213	GRCtrDsFVr GLSFARSG.. GDWAECFA~~	~~
A. niger NRRL3135	GRCtrDsFVr GLSFARSG.. GDWAECFA~~	~~
A. fumigatus ATCC13073	GRCKLNDFVK GLSWARSG.. GNWGECFS~~	~~
A. fumigatus ATCC32722	GRCKLNDFVK GLSWARSG.. GNWGECFS~~	~~
A. fumigatus ATCC58128	GRCKLNDFVK GLSWARSG.. GNWGECFS~~	~~
A. fumigatus ATCC26906	GRCKLNDFVK GLSWARSG.. GNWGECFS~~	~~
A. fumigatus ATCC32239	GRCKLKDFVK GLSWARSG.. GNSEQSFS~~	~~
E. nidulans	GRCtLDDWVE GLNFARSG.. GNWktCFTl~	~~
T. thermophilus	GRCKrDDFVr GLSFARqG.. GNWEGCYAas	e~
T. lanuginosa	GRCRrDEWIK GLTFARqG.. GHWDrCF~~~	~~
M. thermophila	GmCtLErFIE SMAFARGN.. GKWDlCFA~~	~~
Consensus Seq. 11	**GRCKLDDFVE GLSFARSG-- GNWAECFA--**	**--**

Figure 19

```
          M  G  V  F  V  V  L  L  S  I  A  T  L  F  G  S  T  S  G  T
20
          ATGGGCGTGTTCGTCGTGCTACTGTCCATTGCCACCTTGTTCGGTTCCACATCCGGTACC
        1 ---+---------+---------+---------+---------+---------+---------+------
60
          TACCCGCACAAGCAGCACGATGACAGGTAACGGTGGAACAAGCCAAGGTGTAGGCCATGG

          A  L  G  P  R  G  N  S  H  S  C  D  T  V  D  G  G  Y  Q  C
40
          GCCTTGGGTCCTCGTGGTAATTCTCACTCTTGTGACACTGTTGACGGTGGTTACCAATGT
       61 ---+---------+---------+---------+---------+---------+---------+------
120
          CGGAACCCAGGAGCACCATTAAGAGTGAGAACACTGTGACAACTGCCACCAATGGTTACA

          F  P  E  I  S  H  L  W  G  T  Y  S  P  Y  F  S  L  A  D  E
60
          TTCCCAGAAATTTCTCACTTGTGGGGTACCTACTCTCCATACTTCTCTTTGGCAGACGAA
      121 ---+---------+---------+---------+---------+---------+---------+------
180
          AAGGGTCTTTAAAGAGTGAACACCCCATGGATGAGAGGTATGAAGAGAAACCGTCTGCTT

          S  A  I  S  P  D  V  P  D  D  C  R  V  T  F  V  Q  V  L  S
80
          TCTGCTATTTCTCCAGACGTTCCAGACGACTGTAGAGTTACTTTCGTTCAAGTTTTGTCT
      187 ---+---------+---------+---------+---------+---------+---------+------
240
          AGACGATAAAGAGGTCTGCAAGGTCTGCTGACATCTCAATGAAAGCAAGTTCAAAACAGA

          R  H  G  A  R  Y  P  T  S  S  A  S  K  A  Y  S  A  L  I  E
100
          AGACACGGTGCTAGATACCCAACTTCTTCTGCGTCTAAGGCTTACTCTGCTTTGATTGAA
      241 ---+---------+---------+---------+---------+---------+---------+------
300
          TCTGTGCCACGATCTATGGGTTGAAGAAGACGCAGATTCCGAATGAGACGAAACTAACTT

          A  I  Q  K  N  A  T  A  F  K  G  K  Y  A  F  L  K  T  Y  N
120
          GCTATTCAAAAGAACGCTACTGCTTTCAAGGGTAAGTACGCTTTCTTGAAGACTTACAAC
      301 ---+---------+---------+---------+---------+---------+---------+------
360
          CGATAAGTTTTCTTGCGATGACGAAAGTTCCCATTCATGCGAAAGAACTTCTGAATGTTG

          Y  T  L  G  A  D  D  L  T  P  F  G  E  N  Q  M  V  N  S  G
140
          TACACTTTGGGTGCTGACGACTTGACTCCATTCGGTGAAAACCAAATGGTTAACTCTGGT
      361 ---+---------+---------+---------+---------+---------+---------+------
420
          ATGTGAAACCCACGACTGCTGAACTGAGGTAAGCCACTTTTGGTTTACCAATTGAGACCA

          I  K  F  Y  R  R  Y  K  A  L  A  R  K  I  V  P  F  I  R  A
160
          ATTAAGTTCTACAGAAGATACAAGGCTTTGGCTAGAAAGATTGTTCCATTCATTAGAGCT
      421 ---+---------+---------+---------+---------+---------+---------+------
480
          TAATTCAAGATGTCTTCTATGTTCCGAAACCGATCTTTCTAACAAGGTAAGTAATCTCGA

          S  G  S  D  R  V  I  A  S  A  E  K  F  I  E  G  F  Q  S  A
180
          TCTGGTTCTGACAGAGTTATTGCTTCTGCTGAAAAGTTCATTGAAGGTTTCCAATCTGCT
```

```
      481 ---+---------+---------+---------+---------+---------+---------+------
540
          AGACCAAGACTGTCTCAATAACGAAGACGACTTTTCAAGTAACTTCCAAAGGTTAGACGA

          K  L  A  D  P  G  S  Q  P  H  Q  A  S  P  V  I  N  V  I  I
200
          AAGTTGGCTGACCCAGGTTCTCAACCACACCAAGCTTCTCCAGTTATTAACGTGATCATT
      541 ---+---------+---------+---------+---------+---------+---------+------
600
          TTCAACCGACTGGGTCCAAGAGTTGGTGTGGTTCGAAGAGGTCAATAATTGCACTAGTAA

          P  E  G  S  G  Y  N  N  T  L  D  H  G  T  C  T  A  F  E  D
220
          CCAGAAGGATCCGGTTACAACAACACTTTGGACCACGGTACTTGTACTGCTTTCGAAGAC
      601 ---+---------+---------+---------+---------+---------+---------+------
660
          GGTCTTCCTAGGCCAATGTTGTTGTGAAACCTGGTGCCATGAACATGACGAAAGCTTCTG

          S  E  L  G  D  D  V  E  A  N  F  T  A  L  F  A  P  A  I  R
240
          TCTGAATTAGGTGACGACGTTGAAGCTAACTTCACTGCTTTGTTCGCTCCAGCTATTAGA
      661 ---+---------+---------+---------+---------+---------+---------+------
720
          AGACTTAATCCACTGCTGCAACTTCGATTGAAGTGACGAAACAAGCGAGGTCGATAATCT

          A  R  L  E  A  D  L  P  G  V  T  L  T  D  E  D  V  V  Y  L
260
          GCTAGATTGGAAGCTGACTTGCCAGGTGTTACTTTGACTGACGAAGACGTTGTTTACTTG
      721 ---+---------+---------+---------+---------+---------+---------+------
780
          CGATCTAACCTTCGACTGAACGGTCCACAATGAAACTGACTGCTTCTGCAACAAATGAAC

          M  D  M  C  P  F  D  T  V  A  R  T  S  D  A  T  E  L  S  P
280
          ATGGACATGTGTCCATTCGACACTGTCGCTAGAACTTCTGACGCTACTGAATTGTCTCCA
      781 ---+---------+---------+---------+---------+---------+---------+------
840
          TACCTGTACACAGGTAAGCTGTGACAGCGATCTTGAAGACTGCGATGACTTAACAGAGGT

          F  C  A  L  F  T  H  D  E  W  I  Q  Y  D  Y  L  Q  S  L  G
300
          TTCTGTGCTTTGTTCACTCACGACGAATGGATCCAATACGACTACTTGCAAAGCTTGGGT
      841 ---+---------+---------+---------+---------+---------+---------+------
900
          AAGACACGAAACAAGTGAGTGCTGCTTACCTAGGTTATGCTGATGAACGTTTCGAACCCA

          K  Y  Y  G  Y  G  A  G  N  P  L  G  P  A  Q  G  V  G  F  A
320
          AAGTACTACGGTTACGGTGCTGGTAACCCATTGGGTCCAGCTCAAGGTGTTGGTTTCGCT
      901 ---+---------+---------+---------+---------+---------+---------+------
960
          TTCATGATGCCAATGCCACGACCATTGGGTAACCCAGGTCGAGTTCCACAACCAAAGCGA

          N  E  L  I  A  R  L  T  H  S  P  V  Q  D  H  T  S  T  N  H
340
          AACGAATTGATTGCTAGATTGACTCACTCTCCAGTTCAAGACCACACTTCTACTAACCAC
      961 ---+---------+---------+---------+---------+---------+---------+------
1020
          TTGCTTAACTAACGATCTAACTGAGTGAGAGGTCAAGTTCTGGTGTGAAGATGATTGGTG

          T  L  D  S  N  P  A  T  F  P  L  N  A  T  L  Y  A  D  F  S
360
```

```
          ACTTTGGACTCTAACCCAGCTACTTTCCCATTGAACGCTACTTTGTACGCTGACTTCTCT
     1021 ---+---------+---------+---------+---------+---------+---------+------
1080
          TGAAACCTGAGATTGGGTCGATGAAAGGGTAACTTGCGATGAAACATGCGACTGAAGAGA

          H  D  N  T  M  I  S  I  F  F  A  L  G  L  Y  N  G  T  K  P
380
          CACGACAACACTATGATATCTATTTTCTTCGCTTTGGGTTTGTACAACGGTACCAAGCCA
     1081 ---+---------+---------+---------+---------+---------+---------+------
1140
          GTGCTGTTGTGATACTATAGATAAAAGAAGCGAAACCCAAACATGTTGCCATGGTTCGGT

          L  S  T  T  S  V  E  S  I  E  E  T  D  G  Y  S  A  S  W  T
400
          TTGTCTACTACTTCTGTTGAATCTATTGAAGAAACTGACGGTTACTCTGCTTCTTGGACT
     1141 ---+---------+---------+---------+---------+---------+---------+------
1200
          AACAGATGATGAAGACAACTTAGATAACTTCTTTGACTGCCAATGAGACGAAGAACCTGA

          V  P  F  A  A  R  A  Y  V  E  M  M  Q  C  Q  A  E  K  E  P
420
          GTTCCATTCGCTGCTAGAGCTTACGTTGAAATGATGCAATGTCAAGCTGAAAAGGAACCA
     1201 ---+---------+---------+---------+---------+---------+---------+------
1260
          CAAGGTAAGCGACGATCTCGAATGCAACTTTACTACGTTACAGTTCGACTTTTCCTTGGT

          L  V  R  V  L  V  N  D  R  V  V  P  L  H  G  C  A  V  D  K
440
          TTGGTTAGAGTTTTGGTTAACGACAGAGTTGTTCCATTGCACGGTTGTGCTGTTGACAAG
     1261 ---+---------+---------+---------+---------+---------+---------+------
1320
          AACCAATCTCAAAACCAATTGCTGTCTCAACAAGGTAACGTGCCAACACGACAACTGTTC

          L  G  R  C  K  R  D  D  F  V  E  G  L  S  F  A  R  S  G  G
460
          TTGGGTAGATGTAAGAGAGACGACTTCGTTGAAGGTTTGTCTTTCGCTAGATCTGGTGGT
     1321 ---+---------+---------+---------+---------+---------+---------+------
1380
          AACCCATCTACATTCTCTCTGCTGAAGCAACTTCCAAACAGAAAGCGATCTAGACCACCA

          N  W  A  E  C  F  A  *     467
          AACTGGGCTGAATGTTTCGCTTAA
     1381 ---+---------+---------+ 1410
          TTGACCCGACTTACAAAGCGAATT
```

Figure 20

```
          M  G  V  F  V  V  L  L  S  I  A  T  L  F  G  S  T  S  G  T
20
          ATGGGCGTGTTCGTCGTGCTACTGTCCATTGCCACCTTGTTCGGTTCCACATCCGGTACC
        1 ---------+---------+---------+---------+---------+---------+
60
          TACCCGCACAAGCAGCACGATGACAGGTAACGGTGGAACAAGCCAAGGTGTAGGCCATGG

          A  L  G  P  R  G  N  S  H  S  C  D  T  V  D  G  G  Y  Q  C
40
          GCCTTGGGTCCTCGTGGTAACTCTCACTCTTGTGACACTGTTGACGGTGGTTACCAATGT
       61 ---------+---------+---------+---------+---------+---------+
120
          CGGAACCCAGGAGCACCATTGAGAGTGAGAACACTGTGACAACTGCCACCAATGGTTACA

A         F  P  E  I  S  H  L  W  G  T  Y  S  P  F  F  S  L  A  D  E
60
          TTCCCAGAAATTTCTCACTTGTGGGGTACATACTCTCCATTCTTCTCTTTGGCTGACGAA
      121 ---------+---------+---------+---------+---------+---------+
180
          AAGGGTCTTTAAAGAGTGAACACCCCATGTATGAGAGGTAAGAAGAGAAACCGACTGCTT

          S  A  I  S  P  D  V  P  K  G  C  R  V  T  F  V  Q  V  L  S
80
          TCTGCTATTTCTCCAGACGTTCCAAAGGGTTGTAGAGTTACTTTCGTTCAAGTTTTGTCT
      181 ---------+---------+---------+---------+---------+---------+
240
          AGACGATAAAGAGGTCTGCAAGGTTTCCCAACATCTCAATGAAAGCAAGTTCAAAACAGA

          R  H  G  A  R  Y  P  T  S  S  A  S  K  A  Y  S  A  L  I  E
100
          AGACACGGTGCTAGATACCCAACTTCTTCTGCGTCTAAGGCGTACTCTGCTTTGATTGAA
      241 ---------+---------+---------+---------+---------+---------+
300
          TCTGTGCCACGATCTATGGGTTGAAGAAGACGCAGATTCCGCATGAGACGAAACTAACTT

          A  I  Q  K  N  A  T  A  F  K  G  K  Y  A  F  L  K  T  Y  N
120
          GCTATTCAAAAGAACGCTACTGCTTTCAAGGGTAAGTACGCTTTCTTGAAGACTTACAAC
      301 ---------+---------+---------+---------+---------+---------+
360
          CGATAAGTTTTCTTGCGATGACGAAAGTTCCCATTCATGCGAAAGAACTTCTGAATGTTG

A         Y  T  L  G  A  D  D  L  T  P  F  G  E  Q  Q  M  V  N  S  G
140
          TACACTTTGGGTGCTGACGACTTGACTCCATTCGGTGAACAACAAATGGTTAACTCTGGT
      361 ---------+---------+---------+---------+---------+---------+
420
          ATGTGAAACCCACGACTGCTGAACTGAGGTAAGCCACTTGTTGTTTACCAATTGAGACCA

          I  K  F  Y  R  R  Y  K  A  L  A  R  K  I  V  P  F  I  R  A
160
          ATTAAGTTCTACAGAAGATACAAGGCTTTGGCTAGAAAGATTGTTCCATTCATTAGAGCT
      421 ---------+---------+---------+---------+---------+---------+
480
          TAATTCAAGATGTCTTCTATGTTCCGAAACCGATCTTTCTAACAAGGTAAGTAATCTCGA

          S  G  S  D  R  V  I  A  S  A  E  K  F  I  E  G  F  Q  S  A
180
          TCTGGTTCTGACAGAGTTATTGCTTCTGCTGAAAAGTTCATTGAAGGTTTCCAATCTGCT
```

```
    481 ---------+---------+---------+---------+---------+---------+
540
        AGACCAAGACTGTCTCAATAACGAAGACGACTTTTCAAGTAACTTCCAAAGGTTAGACGA
           K  L  A  D  P  G  A  N  P  H  Q  A  S  P  V  I  N  V  I  I
200
        AAGTTGGCTGACCCAGGTGCTAACCCACACCAAGCTTCTCCAGTTATTAACGTTATTATT
    541 ---------+---------+---------+---------+---------+---------+
600
        TTCAACCGACTGGGTCCACGATTGGGTGTGGTTCGAAGAGGTCAATAATTGCAATAATAA
           P  E  G  A  G  Y  N  N  T  L  D  H  G  L  C  T  A  F  E  E
220
        CCAGAAGGTGCTGGTTACAACAACACTTTGGACCACGGTTTGTGTACTGCTTTCGAAGAA
    601 ---------+---------+---------+---------+---------+---------+
660
        GGTCTTCCACGACCAATGTTGTTGTGAAACCTGGTGCCAAACACATGACGAAAGCTTCTT
           S  E  L  G  D  D  V  E  A  N  F  T  A  V  F  A  P  P  I  R
240
        TCTGAATTGGGTGACGACGTTGAAGCTAACTTCACTGCTGTTTTCGCTCCACCAATTAGA
    661 ---------+---------+---------+---------+---------+---------+
720
        AGACTTAACCCACTGCTGCAACTTCGATTGAAGTGACGACAAAAGCGAGGTGGTTAATCT
           A  R  L  E  A  H  L  P  G  V  N  L  T  D  E  D  V  V  N  L
260
        GCTAGATTGGAAGCTCACTTGCCAGGTGTTAACTTGACTGACGAAGACGTTGTTAACTTG
    721 ---------+---------+---------+---------+---------+---------+
780
        CGATCTAACCTTCGAGTGAACGGTCCACAATTGAACTGACTGCTTCTGCAACAATTGAAC
           M  D  M  C  P  F  D  T  V  A  R  T  S  D  A  T  Q  L  S  P
280
        ATGGACATGTGTCCATTCGACACTGTTGCTAGAACTTCTGACGCTACTCAATTGTCTCCA
    781 ---------+---------+---------+---------+---------+---------+
840
        TACCTGTACACAGGTAAGCTGTGACAACGATCTTGAAGACTGCGATGAGTTAACAGAGGT
           F  C  D  L  F  T  H  D  E  W  I  Q  Y  D  Y  L  Q  S  L  G
300
        TTCTGTGACTTGTTCACTCACGACGAATGGATTCAATACGACTACTTGCAATCTTTGGGT
    841 ---------+---------+---------+---------+---------+---------+
900
        AAGACACTGAACAAGTGAGTGCTGCTTACCTAAGTTATGCTGATGAACGTTAGAAACCCA
           K  Y  Y  G  Y  G  A  G  N  P  L  G  P  A  Q  G  V  G  F  V
320
        AAGTACTACGGTTACGGTGCTGGTAACCCATTGGGTCCAGCTCAAGGTGTTGGTTTCGTT
    901 ---------+---------+---------+---------+---------+---------+
960
        TTCATGATGCCAATGCCACGACCATTGGGTAACCCAGGTCGAGTTCCACAACCAAAGCAA
           N  E  L  I  A  R  L  T  H  S  P  V  Q  D  H  T  S  T  N  H
340
        AACGAATTGATTGCTAGATTGACTCACTCTCCAGTTCAAGACCACACTTCTACTAACCAC
    961 ---------+---------+---------+---------+---------+---------+
1020
        TTGCTTAACTAACGATCTAACTGAGTGAGAGGTCAAGTTCTGGTGTGAAGATGATTGGTG
           T  L  D  S  N  P  A  T  F  P  L  N  A  T  L  Y  A  D  F  S
360
```

```
        ACTTTGGACTCTAACCCAGCTACTTTCCCATTGAACGCTACTTTGTACGCTGACTTCTCT
    1021 ---------+---------+---------+---------+---------+---------+
1080
        TGAAACCTGAGATTGGGTCGATGAAAGGGTAACTTGCGATGAAACATGCGACTGAAGAGA

        H  D  N  T  M  V  S  I  F  F  A  L  G  L  Y  N  G  T  K  P
380
        CACGACAACACTATGGTTTCTATTTTCTTCGCTTTGGGTTTGTACAACGGTACTAAGCCA
    1081 ---------+---------+---------+---------+---------+---------+
1140
        GTGCTGTTGTGATACCAAAGATAAAAGAAGCGAAACCCAAACATGTTGCCATGATTCGGT

        L  S  T  T  S  V  E  S  I  E  E  T  D  G  Y  S  A  S  W  T
400
        TTGTCTACTACTTCTGTTGAATCTATTGAAGAAACTGACGGTTACTCTGCTTCTTGGACT
    1141 ---------+---------+---------+---------+---------+---------+
1200
        AACAGATGATGAAGACAACTTAGATAACTTCTTTGACTGCCAATGAGACGAAGAACCTGA

        V  P  F  A  A  R  A  Y  V  E  M  M  Q  C  E  A  E  K  E  P
420
        GTTCCATTCGCTGCTAGAGCTTACGTTGAAATGATGCAATGTGAAGCTGAAAAGGAACCA
    1201 ---------+---------+---------+---------+---------+---------+
1260
        CAAGGTAAGCGACGATCTCGAATGCAACTTTACTACGTTACACTTCGACTTTTCCTTGGT

        L  V  R  V  L  V  N  D  R  V  V  P  L  H  G  C  G  V  D  K
440
        TTGGTTAGAGTTTTGGTTAACGACAGAGTTGTTCCATTGCACGGTTGTGGTGTTGACAAG
    1261 ---------+---------+---------+---------+---------+---------+
1320
        AACCAATCTCAAAACCAATTGCTGTCTCAACAAGGTAACGTGCCAACACCACAACTGTTC

        L  G  R  C  K  R  D  D  F  V  E  G  L  S  F  A  R  S  G  G
460
        TTGGGTAGATGTAAGAGAGACGACTTCGTTGAAGGTTTGTCTTTCGCTAGATCTGGTGGT
    1321 ---------+---------+---------+---------+---------+---------+
1380
        AACCCATCTACATTCTCTCTGCTGAAGCAACTTCCAAACAGAAAGCGATCTAGACCACCA

        N  W  E  E  C  F  A  *     467
        AACTGGGAAGAATGTTTCGCTTAA
    1381 ---------+---------+---- 1404
        TTGACCCTTCTTACAAAGCGAATT
```

Figure 21

```
          M  G  V  F  V  V  L  L  S  I  A  T  L  F  G  S  T  S  G  T   20
          ATGGGGGTTTTCGTCGTTCTATTATCTATCGCGACTCTGTTCGGCAGCACATCGGGCACT
        1 ---------+---------+---------+---------+---------+---------+ 60
          TACCCCCAAAAGCAGCAAGATAATAGATAGCGCTGAGACAAGCCGTCGTGTAGCCCGTGA

          A  L  G  P  R  G  N  H  S  K  S  C  D  T  V  D  L  G  Y  Q   40
          GCGCTGGGCCCCCGTGGAAATCACTCCAAGTCCTGCGATACGGTAGACCTAGGGTACCAG
       61 ---------+---------+---------+---------+---------+---------+
120
          CGCGACCCGGGGGCACCTTTAGTGAGGTTCAGGACGCTATGCCATCTGGATCCCATGGTC

          C  S  P  A  T  S  H  L  W  G  T  Y  S  P  Y  F  S  L  E  D   60
          TGCTCCCCTGCGACTTCTCATCTATGGGGCACGTACTCGCCATaCTTTTCGCTCGAGGAC
      121 ---------+---------+---------+---------+---------+---------+
180
          ACGAGGGGACGCTGAAGAGTAGATACCCCGtgCATGAGCGGTAtGAAAAGCGAGCTCCTG

          E  L  S  V  S  S  K  L  P  K  D  C  R  I  T  L  V  Q  V  L   80
          GAGCTGTCCGTGTCGAGTAAGCTTCCCAAGGATTGCCGGATCACCTTGGTACAGGTGCTA
      181 ---------+---------+---------+---------+---------+---------+
240
          CTCGACAGGCACAGCTCATTCGAAGGGTTCCTAACGGCCTAGTGGAACCATGTCCACGAT

          S  R  H  G  A  R  Y  P  T  S  S  K  S  K  K  Y  K  K  L  I
100
          TCGCGCCATGGAGCGCGGTACCCAACCAGCTCCAAGAGCAAAAAGTATAAGAAGCTTaTt
      241 ---------+---------+---------+---------+---------+---------+
300
          AGCGCGGTACCTCGCGCCATGGGTTGGTCGAGGTTCTCGTTTTTCATATTCTTCGAAtAa

          T  A  I  Q  A  N  A  T  D  F  K  G  K  Y  A  F  L  K  T  Y
120
          ACGGCGATCCAGGCCAATGCCACCGACTTCAAGGGCAAGTacGCCTTTTTGAAGACGTAC
      301 ---------+---------+---------+---------+---------+---------+
360
          TGCCGCTAGGTCCGGTTACGGTGGCTGAAGTTCCCGTTCAtgCGGAAAAACTTCTGCATG

          N  Y  T  L  G  A  D  D  L  T  P  F  G  E  Q  Q  L  V  N  S
140
          AACTATACTCTGGGTGCGGATGACCTCACTCCCTTTGGGGAGCAGCAGCTGGTGAACTCG
      361 ---------+---------+---------+---------+---------+---------+
420
          TTGATATGAGACCCACGCCTACTGGAGTGAGGGAAACCCCTCGTCGTCGACCACTTGAGC

          G  I  K  F  Y  Q  R  Y  K  A  L  A  R  S  V  V  P  F  I  R
160
          GGCATCAAGTTCTACCAGAGGTACAAGGCTCTGGCGCGCAGTGTGGTGCCGTTTATTCGC
      421 ---------+---------+---------+---------+---------+---------+
480
          CCGTAGTTCAAGATGGTCTCCATGTTCCGAGACCGCGCGTCACACCACGGCAAATAAGCG

          A  S  G  S  D  R  V  I  A  S  G  E  K  F  I  E  G  F  Q  Q
180
          GCCTCAGGCTCGGACCGGGTTATTGCTTCGGGAGAGAAGTTCATCGAGGGGTTCCAGCAG
      481 ---------+---------+---------+---------+---------+---------+
540
          CGGAGTCCGAGCCTGGCCCAATAACGAAGCCCTCTCTTCAAGTAGCTCCCCAAGGTCGTC

          A  K  L  A  D  P  G  A  T  N  R  A  A  P  A  I  S  V  I  I
200
```

```
      GCGAAGCTGGCTGATCCTGGCGCGACGAACCGCGCCGCTCCGGCGATTAGTGTGATTATT
  541 ---------+---------+---------+---------+---------+---------+
600
      CGCTTCGACCGACTAGGACCGCGCTGCTTGGCGCGGCGAGGCCGCTAATCACACTAATAA

      P  E  S  E  T  F  N  N  T  L  D  H  G  V  C  T  K  F  E  A
220
      CCGGAGAGCGAGACGTTCAACAATACGCTGGACCACGGTGTGTGCACGAAGTTTGAGGCG
  601 ---------+---------+---------+---------+---------+---------+
660
      GGCCTCTCGCTCTGCAAGTTGTTATGCGACCTGGTGCCACACACGTGCTTCAAACTCCGC

      S  Q  L  G  D  E  V  A  A  N  F  T  A  L  F  A  P  D  I  R
240
      AGTCAGCTGGGAGATGAGGTTGCGGCCAATTTCACTGCGCTCTTTGCACCCGACATCCGA
  661 ---------+---------+---------+---------+---------+---------+
720
      TCAGTCGACCCTCTACTCCAACGCCGGTTAAAGTGACGCGAGAAACGTGGGCTGTAGGCT

      A  R  L  E  K  H  L  P  G  V  T  L  T  D  E  D  V  V  S  L
260
      GCTCGCctCGAGAAGCATCTTCCTGGCGTGACGCTGACAGACGAGGACGTTGTCAGTCTA
  721 ---------+---------+---------+---------+---------+---------+
780
      CGAGCGgaGCTCTTCGTAGAAGGACCGCACTGCGACTGTCTGCTCCTGCAACAGTCAGAT

      M  D  M  C  P  F  D  T  V  A  R  T  S  D  A  S  Q  L  S  P
280
      ATGGACATGTGTcCGTTTGATACGGTAGCGCGCACCAGCGACGCAAGTCAGCTGTCACCG
  781 ---------+---------+---------+---------+---------+---------+
840
      TACCTGTACACAgGCAAACTATGCCATCGCGCGTGGTCGCTGCGTTCAGTCGACAGTGGC

      F  C  Q  L  F  T  H  N  E  W  K  K  Y  D  Y  L  Q  S  L  G
300
      TTCTGTCAACTCTTCACTCACAATGAGTGGAAGAAGTACgACTACCTTCAGTCCTTGGGC
  841 ---------+---------+---------+---------+---------+---------+
900
      AAGACAGTTGAGAAGTGAGTGTTACTCACCTTCTTCATGcTGATGGAAGTCAGGAACCCG

      K  Y  Y  G  Y  G  A  G  N  P  L  G  P  A  Q  G  I  G  F  T
320
      AAGTACTACGGCTACGGCGCAGGCAACCCTCTGGGACCGGCTCAGGGGATAGGGTTCACC
  901 ---------+---------+---------+---------+---------+---------+
960
      TTCATGATGCCGATGCCGCGTCCGTTGGGAGACCCTGGCCGAGTCCCCTATCCCAAGTGG

      N  E  L  I  A  R  L  T  R  S  P  V  Q  D  H  T  S  T  N  S
340
      AACGAGCTGATTGCCCGGTTGACgCGTTCGCCAGTGCAGGACCACACCAGCACTAACTCG
  961 ---------+---------+---------+---------+---------+---------+
1020
      TTGCTCGACTAACGGGCCAACTGcGCAAGCGGTCACGTCCTGGTGTGGTCGTGATTGAGC

      T  L  V  S  N  P  A  T  F  P  L  N  A  T  M  Y  V  D  F  S
360
      ACTCTAGTCTCCAACCCGGCCACCTTCCCGTTGAACGCTACCATGTACGTCGACTTTTCA
 1021 ---------+---------+---------+---------+---------+---------+
1080
      TGAGATCAGAGGTTGGGCCGGTGGAAGGGCAACTTGCGATGGTACATGCAGCTGAAAAGT
```

```
        H  D  N  S  M  V  S  I  F  F  A  L  G  L  Y  N  G  T  E  P
380
        CACGACAACAGCATGGTTTCCATCTTCTTTGCATTGGGCCTGTACAACGGCACTGAACCC
    1081 ---------+---------+---------+---------+---------+---------+
1140
        GTGCTGTTGTCGTACCAAAGGTAGAAGAAACGTAACCCGGACATGTTGCCGTGACTTGGG

        L  S  R  T  S  V  E  S  A  K  E  L  D  G  Y  S  A  S  W  V
400
        TTGTCCCGGACCTCGGTGGAAAGCGCCAAGGAATTGGATGGGTATTCTGCATCCTGGGTG
    1141 ---------+---------+---------+---------+---------+---------+
1200
        AACAGGGCCTGGAGCCACCTTTCGCGGTTCCTTAACCTACCCATAAGACGTAGGACCCAC

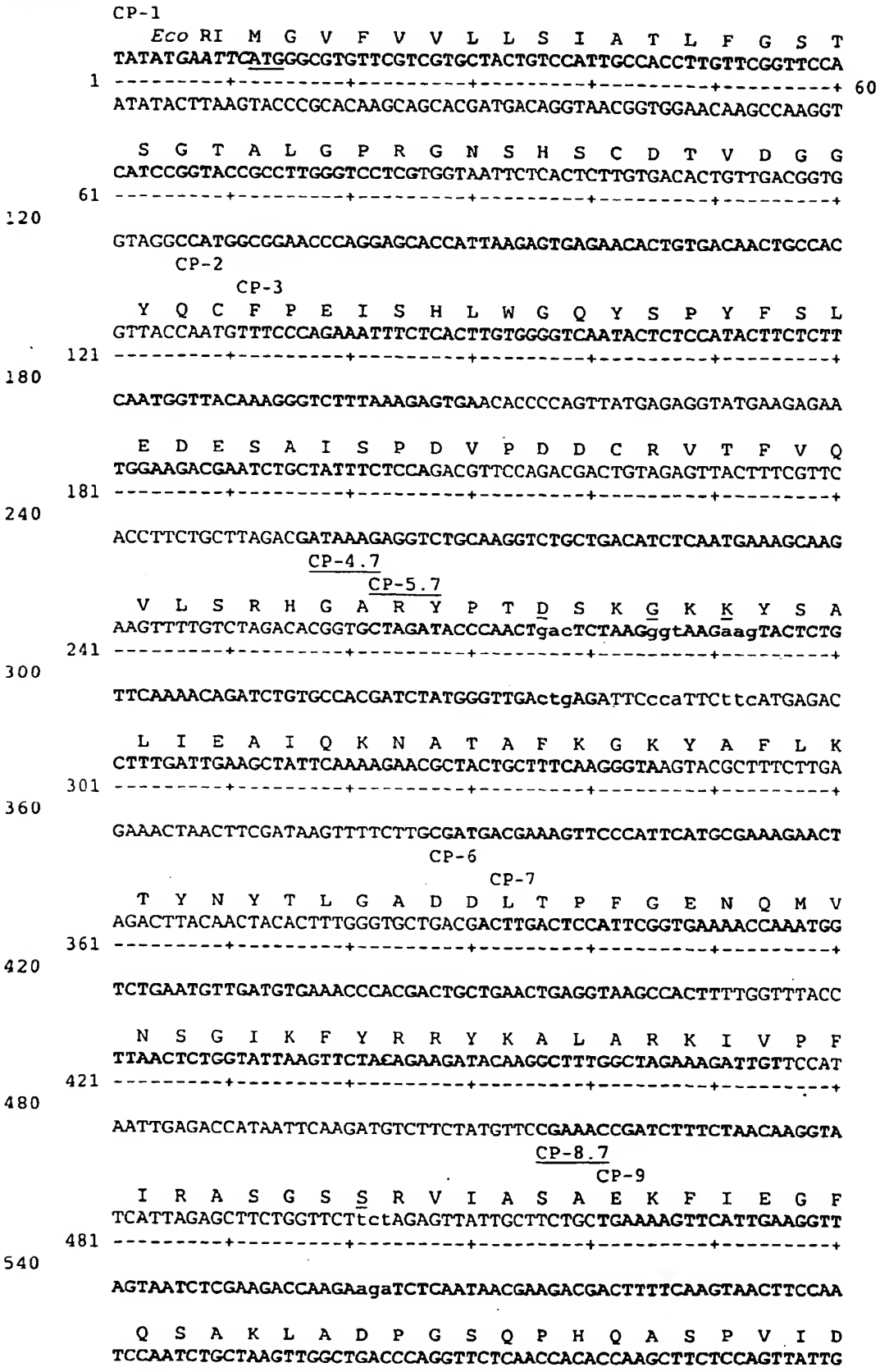
        V  P  F  G  A  R  A  Y  F  E  T  M  Q  C  K  S  E  K  E  P
420
        GTGCCTTTCGGCGCGCGAGCCTACTTCGAGACGATGCAATGCAAGTCGGAAAAGGAGCCT
    1201 ---------+---------+---------+---------+---------+---------+
1260
        CACGGAAAGCCGCGCGCTCGGATGAAGCTCTGCTACGTTACGTTCAGCCTTTTCCTCGGA

        L  V  R  A  L  I  N  D  R  V  V  P  L  H  G  C  D  V  D  K
440
        CTTGTTCGCGCTTTGATTAATGACCGGGTTGTGCCACTGCATGGCTGCGATGTGGACAAG
    1261 ---------+---------+---------+---------+---------+---------+
1320
        GAACAAGCGCGAAACTAATTACTGGCCCAACACGGTGACGTACCGACGCTACACCTGTTC

        L  G  R  C  K  L  N  D  F  V  K  G  L  S  W  A  R  S  G  G
460
        CTGGGGCGATGCAAGCTGAATGACTTTGTCAAGGGATTGAGTTGGGCCAGATCTGGGGGC
    1321 ---------+---------+---------+---------+---------+---------+
1380
        GACCCCGCTACGTTCGACTTACTGAAACAGTTCCCTAACTCAACCCGGTCTAGACCCCCG

        N  W  G  E  C  F  S  *    467
        AACTGGGGAGAGTGCTTTAGTTGA
    1381 ---------+---------+---- 1404
        TTGACCCCTCTCACGAAATCAACT
```

Figure 22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S G T A L G P R G N S H S C D T V D G G
CATCCGGTACCGCCTTGGGTCCTCGTGGTAATTCTCACTCTTGTGACACTGTTGACGGTG
61
120
GTAGGCCATGGCGGAACCCAGGAGCACCATTAAGAGTGAGAACACTGTGACAACTGCCAC
CP-2
CP-3
Y Q C F P E I S H L W G Q Y S P Y F S L
GTTACCAATGTTTCCCAGAAATTTCTCACTTGTGGGGTCAATACTCTCCATACTTCTCTT
121
180
CAATGGTTACAAAGGGTCTTTAAAGAGTGAACACCCCAGTTATGAGAGGTATGAAGAGAA
E D E S A I S P D V P D D C R V T F V Q
TGGAAGACGAATCTGCTATTTCTCCAGACGTTCCAGACGACTGTAGAGTTACTTTCGTTC
181
240
ACCTTCTGCTTAGACGATAAAGAGGTCTGCAAGGTCTGCTGACATCTCAATGAAAGCAAG
CP-4.7
CP-5.7
V L S R H G A R Y P T D S K G K K Y S A
AAGTTTTGTCTAGACACGGTGCTAGATACCCAACTgacTCTAAGggtAAGaagTACTCTG
241
300
TTCAAAACAGATCTGTGCCACGATCTATGGGTTGActgAGATTCccaTTCttcATGAGAC
L I E A I Q K N A T A F K G K Y A F L K
CTTTGATTGAAGCTATTCAAAAGAACGCTACTGCTTTCAAGGGTAAGTACGCTTTCTTGA
301
360
GAAACTAACTTCGATAAGTTTTCTTGCGATGACGAAAGTTCCCATTCATGCGAAAGAACT
CP-6
CP-7
T Y N Y T L G A D D L T P F G E N Q M V
AGACTTACAACTACACTTTGGGTGCTGACGACTTGACTCCATTCGGTGAAAACCAAATGG
361
420
TCTGAATGTTGATGTGAAACCCACGACTGCTGAACTGAGGTAAGCCACTTTTGGTTTACC
N S G I K F Y R R Y K A L A R K I V P F
TTAACTCTGGTATTAAGTTCTACAGAAGATACAAGGCTTTGGCTAGAAAGATTGTTCCAT
421
480
AATTGAGACCATAATTCAAGATGTCTTCTATGTTCCGAAACCGATCTTTCTAACAAGGTA
CP-8.7
CP-9
I R A S G S S R V I A S A E K F I E G F
TCATTAGAGCTTCTGGTTCTTctAGAGTTATTGCTTCTGCTGAAAAGTTCATTGAAGGTT
481
540
AGTAATCTCGAAGACCAAGAagaTCTCAATAACGAAGACGACTTTTCAAGTAACTTCCAA
Q S A K L A D P G S Q P H Q A S P V I D
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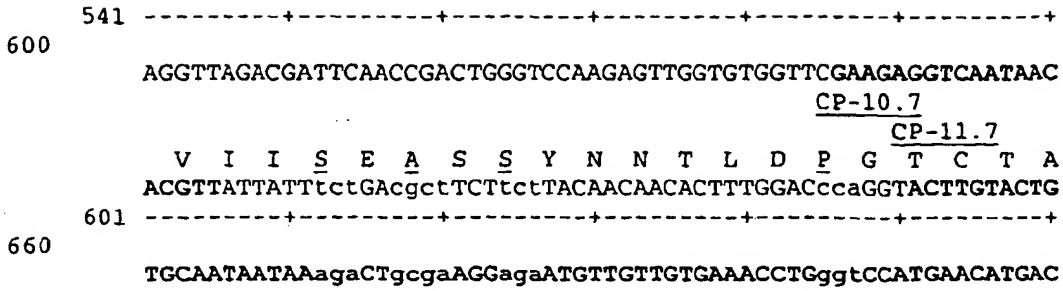

```
          F  E  D  S  E  L  A  D  T  V  E  A  N  F  T  A  L  F  A  P
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     661 ---------+---------+---------+---------+---------+---------+
720

         GAAAGCTTCTGAGACTTAACcgaCTGtgaCAACTTCGATTGAAGTGACGAAACAAGCGAG
                                                              CP-12.7

          A  I  R  A  R  L  E  A  D  L  P  G  V  T  L  T  D  T  E  V
         CAGCTATTAGAGCTAGATTGGAAGCTGACTTGCCAGGTGTTACTTTGACTGACactgaaG
     721 ---------+---------+---------+---------+---------+---------+
780

         GTCGATAATCTCGATCTAACCTTCGACTGAACGGTCCACAATGAAACTGACTGtgacttC

         CP-13.7
          T  Y  L  M  D  M  C  S  F  E  T  V  A  R  T  S  D  A  T  E
         TTactTACTTGATGGACATGTGTtctTTCGAAACTGTTGCTAGAACTTCTGACGCTACTG
     781 ---------+---------+---------+---------+---------+---------+
840

         AAtgaATGAACTACCTGTACACAagaAAGCTTTGACAACGATCTTGAAGACTGCGATGAC

          L  S  P  F  C  A  L  F  T  H  D  E  W  R  H  Y  D  Y  L  Q
         AATTGTCTCCATTCTGTGCTTTGTTCACTCACGACGAATGGAGAcacTACGACTACTTGC
     841 ---------+---------+---------+---------+---------+---------+
900

         TTAACAGAGGTAAGACACGAAACAAGTGAGTGCTGCTTACCTCTgtgATGCTGATGAACG
               CP-14.7
                    CP-15.7
          S  L  K  K  Y  Y  G  H  G  A  G  N  P  L  G  P  T  Q  G  V
         AATCTTTGaagAAGTACTACGGTcacGGTGCTGGTAACCCATTGGGTCCAactCAAGGTG
     901 ---------+---------+---------+---------+---------+---------+
960

         TTAGAAACttcTTCATGATGCCAgtgCCACGACCATTGGGTAACCCAGGTtgaGTTCCAC

          G  F  A  N  E  L  I  A  R  L  T  R  S  P  V  Q  D  H  T  S
         TTGGTTTCGCTAACGAATTGATTGCTAGATTGACTAGATCTCCAGTTCAAGACCACACTT
     961 ---------+---------+---------+---------+---------+---------+
1020

         AACCAAAGCGATTGCTTAACTAACGATCTAACTGATCTAGAGGTCAAGTTCTGGTGTGAA
                         CP-16
                              CP-17.7
          T  N  H  T  L  D  S  N  P  A  T  F  P  L  N  A  T  L  Y  A
         CTACTAACCACACTTTGGACTCTAACCCAGCTACTTTCCCATTGAACGCTACTTTGTACG
    1021 ---------+---------+---------+---------+---------+---------+
1080

         GATGATTGGTGTGAAACCTGAGATTGGGTCGATGAAAGGGTAACTTGCGATGAAACATGC

          D  F  S  H  D  N  G  I  I  S  I  F  F  A  L  G  L  Y  N  G
         CTGACTTCTCTCACGACAACggtattATTTCTATTTTCTTCGCTTTGGGTTTGTACAACG
    1081 ---------+---------+---------+---------+---------+---------+
1140

         GACTGAAGAGAGTGCTGTTGccataaTAAAGATAAAAGAAGCGAAACCCAAACATGTTGC
                                   CP-18.7
                                        CP-19.7
          T  A  P  L  S  T  T  S  V  E  S  I  E  E  T  D  G  Y  S  S
         GTACTGCTCCATTGTCTACTACTTCTGTTGAATCTATTGAAGAAACTGACGGTTACTCTt
    1141 ---------+---------+---------+---------+---------+---------+
1200

         CATGACGAGGTAACAGATGATGAAGACAACTTAGATAACTTCTTTGACTGCCAATGAGAa

          A  W  T  V  P  F  A  S  R  A  Y  V  E  M  M  Q  C  Q  A  E
```

```
        ctgcttGGACTGTTCCATTCgcttctAGAGCTTACGTTGAAATGATGCAATGTCAAGCTG
    1201 ---------+---------+---------+---------+---------+---------+
1260
        gacgaACCTGACAAGGTAAGcgaagaTCTCGAATGCAACTTTACTACGTTACAGTTCGAC
                                           CP-20
                                                CP-21
          K  E  P  L  V  R  V  L  V  N  D  R  V  V  P  L  H  G  C  A
        AAAAGGAACCATTGGTTAGAGTTTTGGTTAACGACAGAGTTGTTCCATTGCACGGTTGTG
    1261 ---------+---------+---------+---------+---------+---------+
1320
        TTTTCCTTGGTAACCAATCTCAAAACCAATTGCTGTCTCAACAAGGTAACGTGCCAACAC
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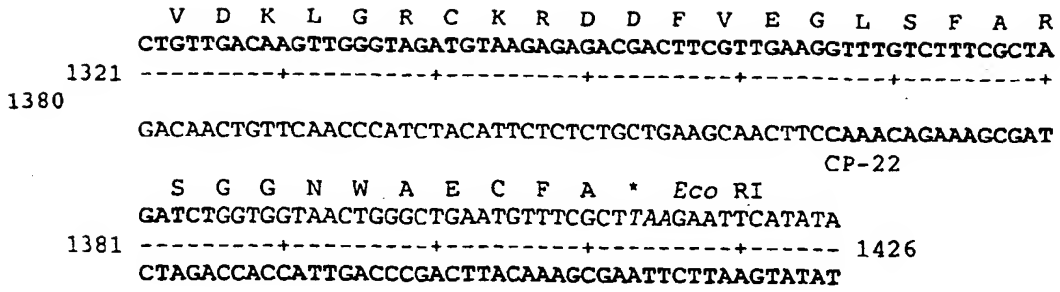
V D K L G R C K R D D F V E G L S F A R
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1321 ---------+---------+---------+---------+---------+---------+
1380
GACAACTGTTCAACCCATCTACATTCTCTCTGCTGAAGCAACTTCCAAACAGAAAGCGAT
CP-22
S G G N W A E C F A * Eco RI
GATCTGGTGGTAACTGGGCTGAATGTTTCGCTTAAGAATTCATATA
1381 ---------+---------+---------+---------+------ 1426
CTAGACCACCATTGACCCGACTTACAAAGCGAATTCTTAAGTATAT

Figure 23

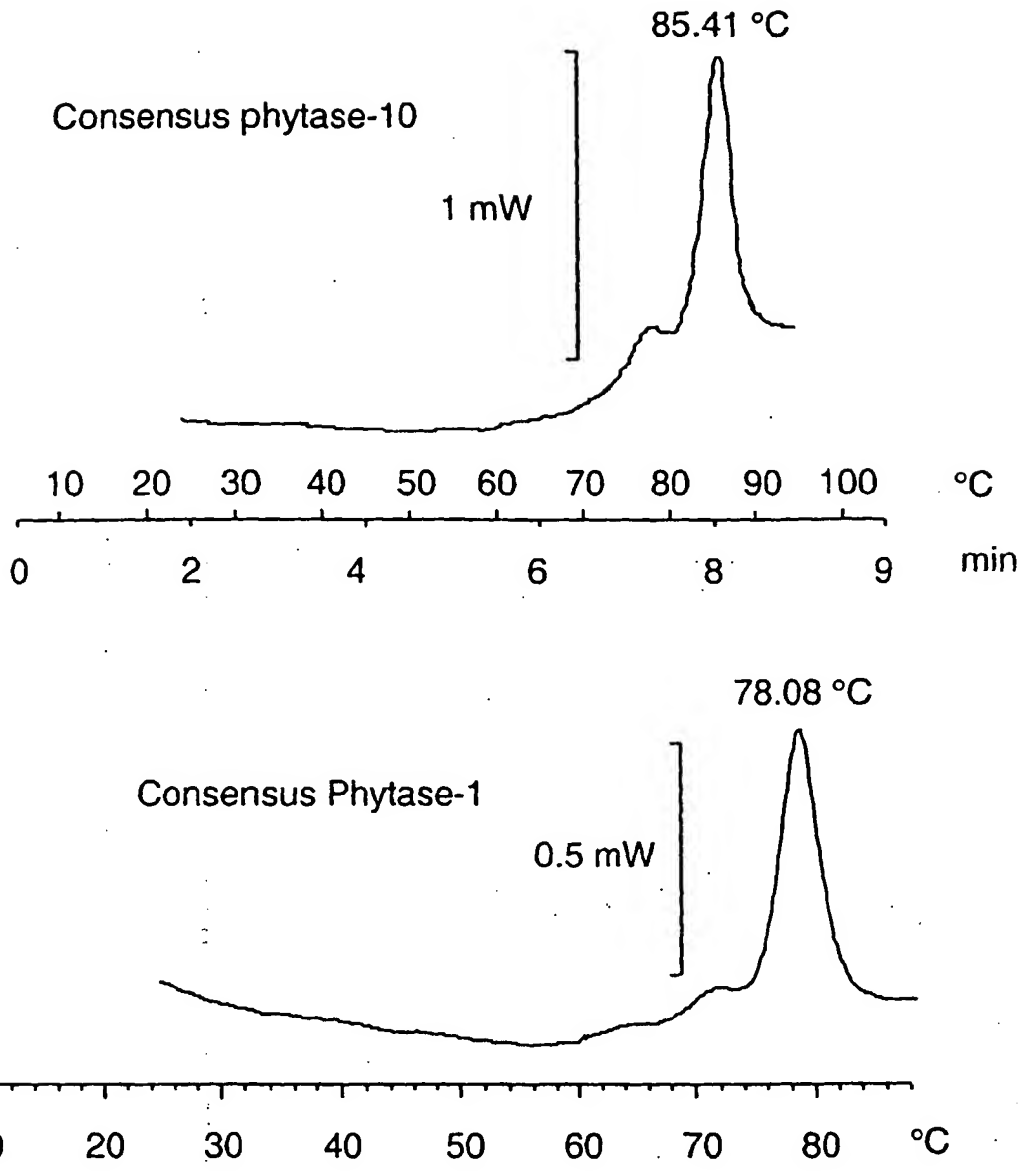

85.41 °C
Consensus phytase-10
1 mW
10 20 30 40 50 60 70 80 90 100 °C
0 2 4 6 8 9 min
78.08 °C
Consensus Phytase-1
0.5 mW
10 20 30 40 50 60 70 80 °C

Figure 24

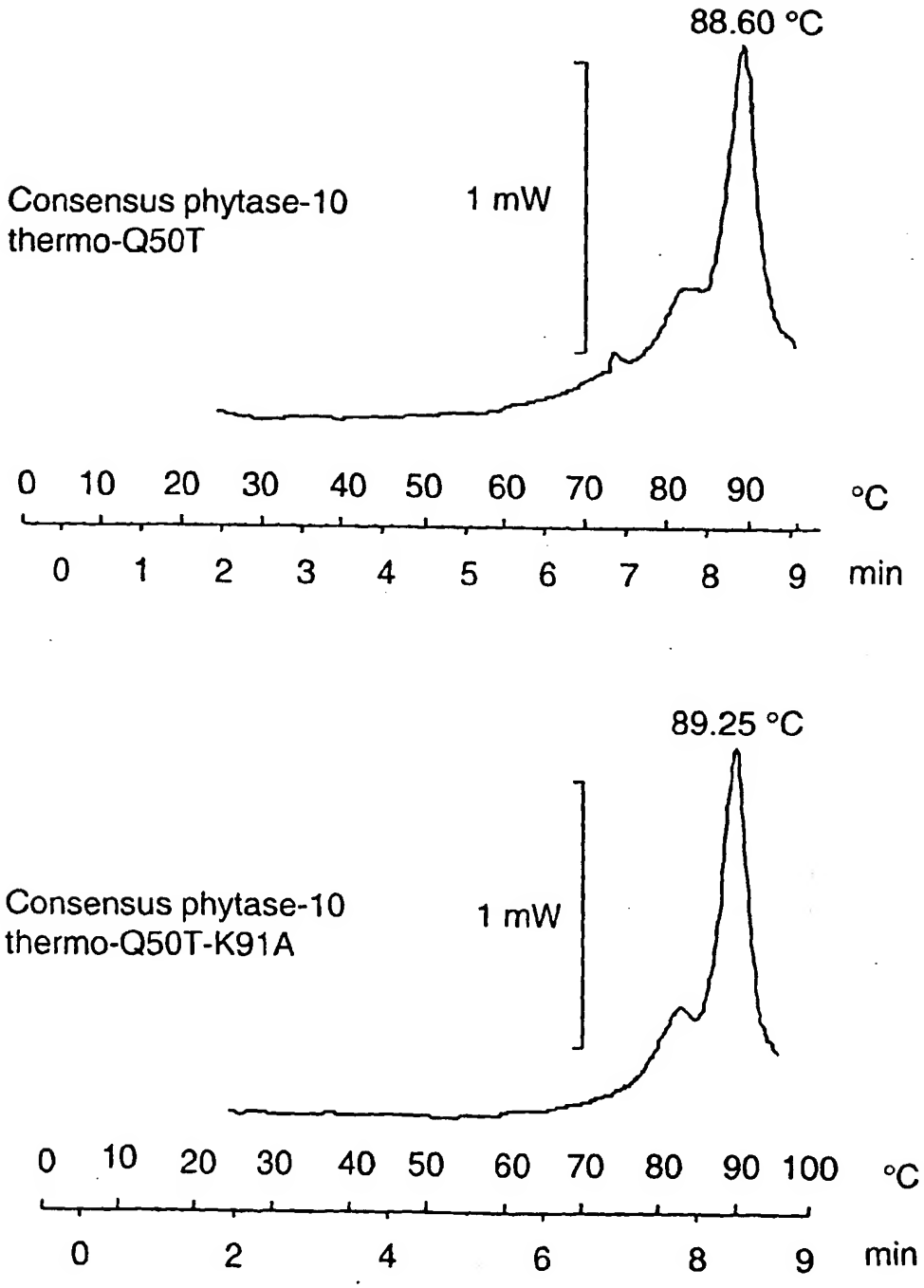

88.60 °C
Consensus phytase-10
thermo-Q50T
1 mW
0 10 20 30 40 50 60 70 80 90 °C
0 1 2 3 4 5 6 7 8 9 min
89.25 °C
Consensus phytase-10
thermo-Q50T-K91A
1 mW
0 10 20 30 40 50 60 70 80 90 100 °C
0 2 4 6 8 9 min

Figure 25

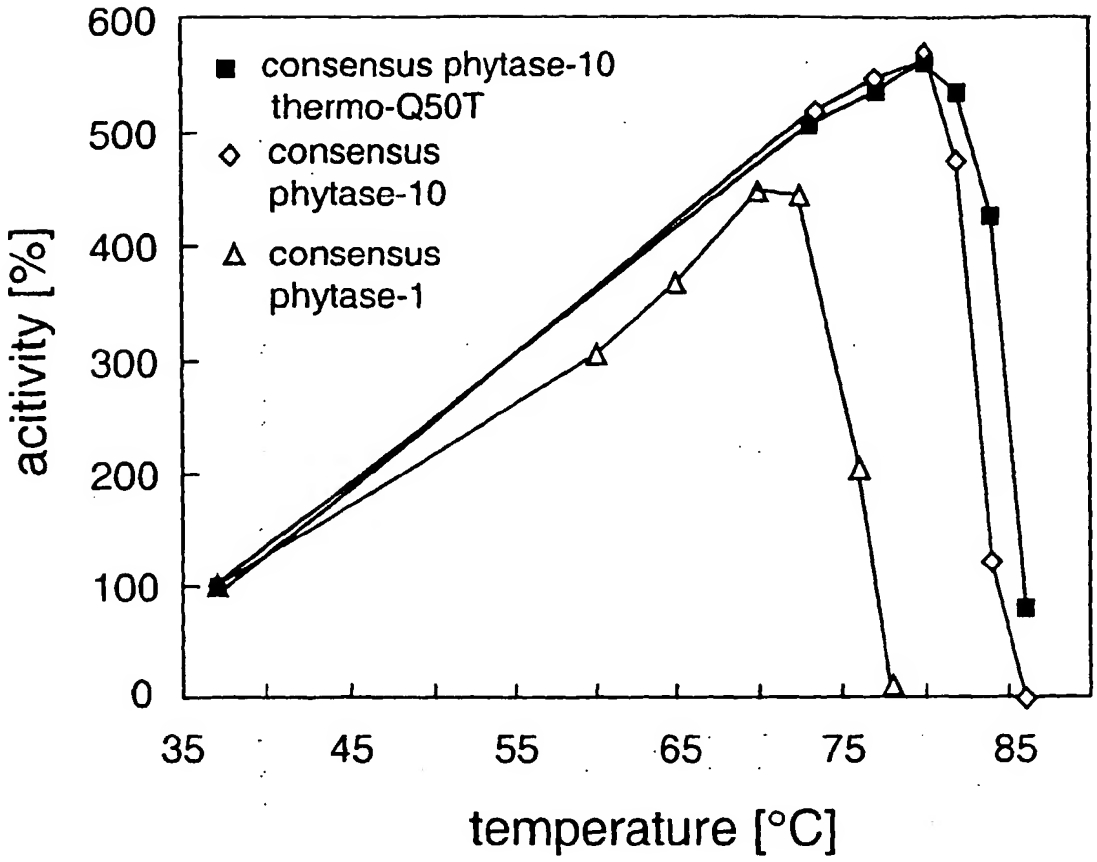


Figure 26

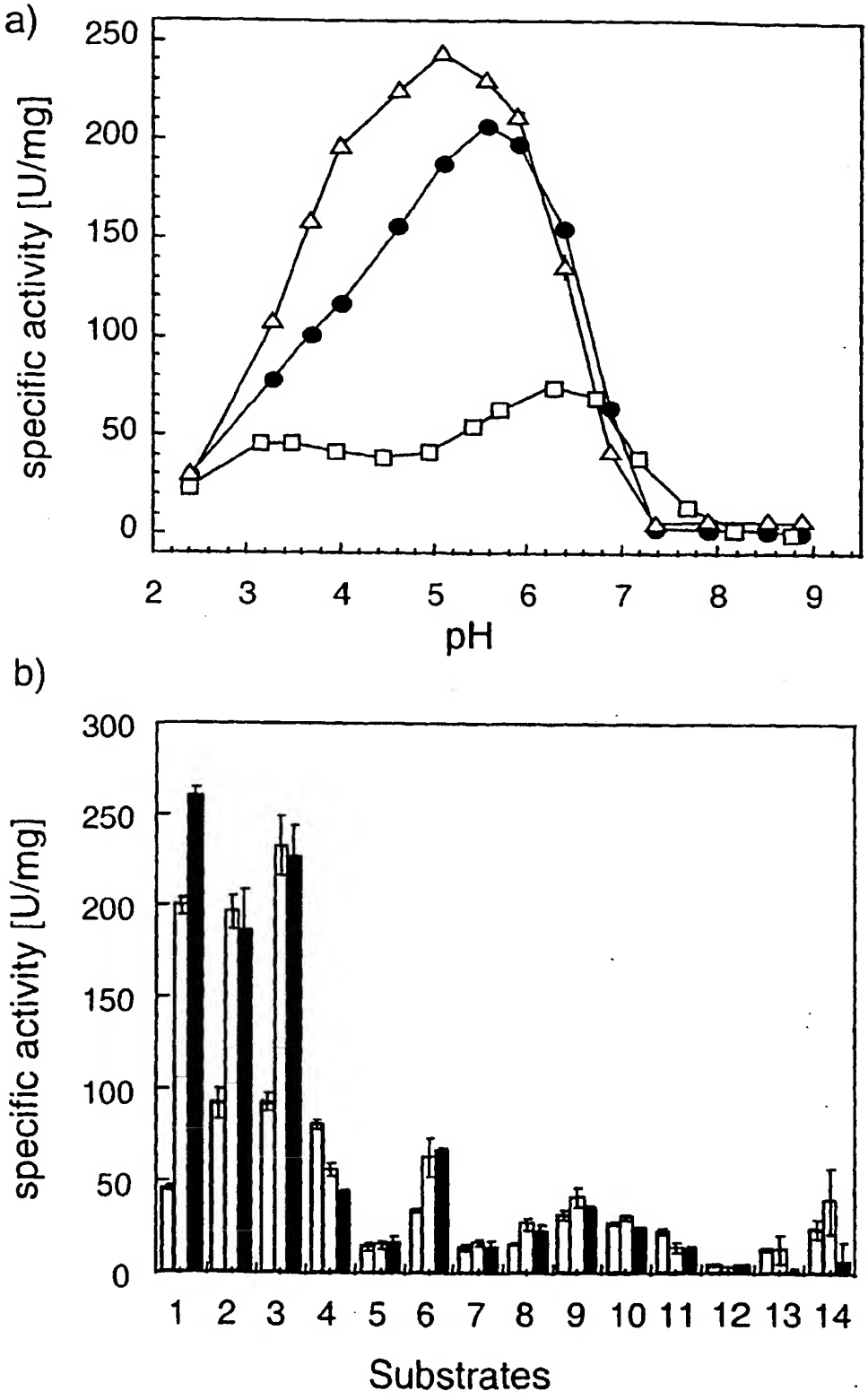
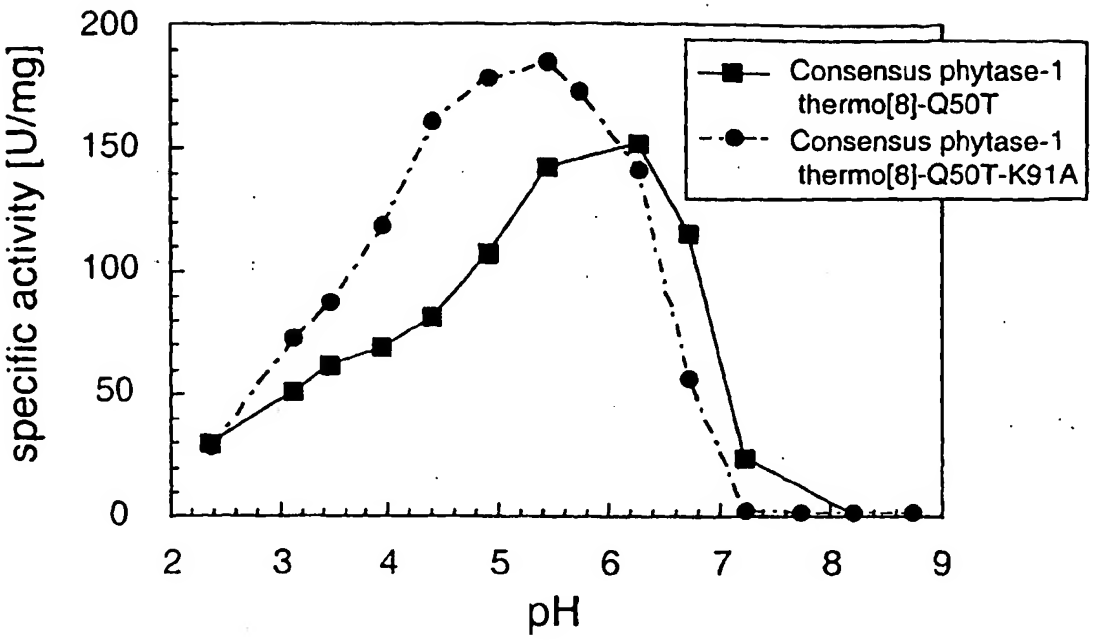

a)
specific activity [U/mg]
250
200
150
100
50
0
2
3
4
5
6
7
8
9
pH
b)
specific activity [U/mg]
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Substrates

Figure 27



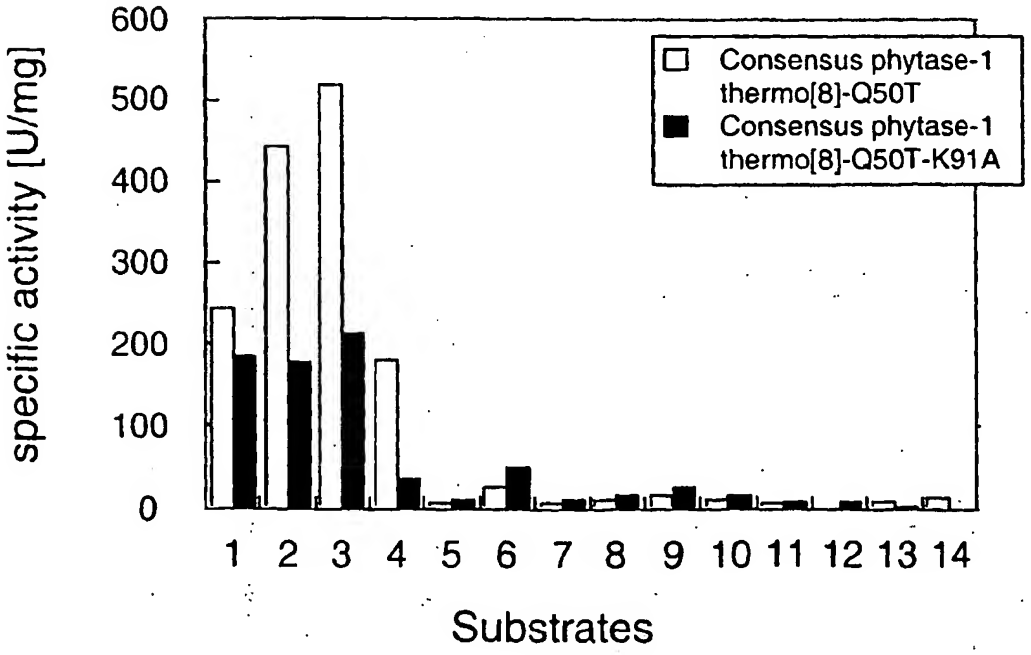


Figure 28

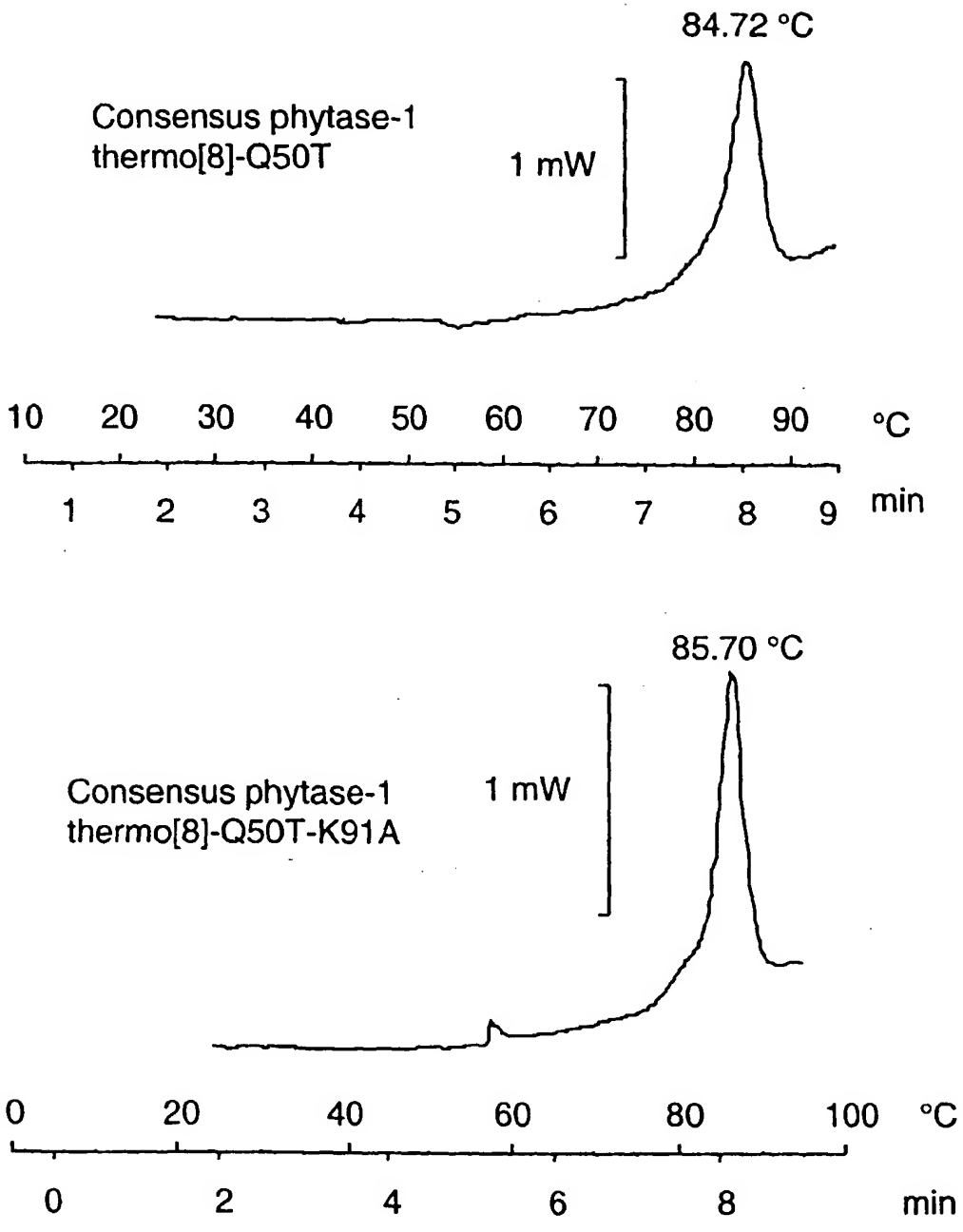

84.72 °C
Consensus phytase-1
thermo[8]-Q50T
1 mW
10 20 30 40 50 60 70 80 90 °C
1 2 3 4 5 6 7 8 9 min
85.70 °C
Consensus phytase-1
thermo[8]-Q50T-K91A
1 mW
0 20 40 60 80 100 °C
0 2 4 6 8 min

Figure 29

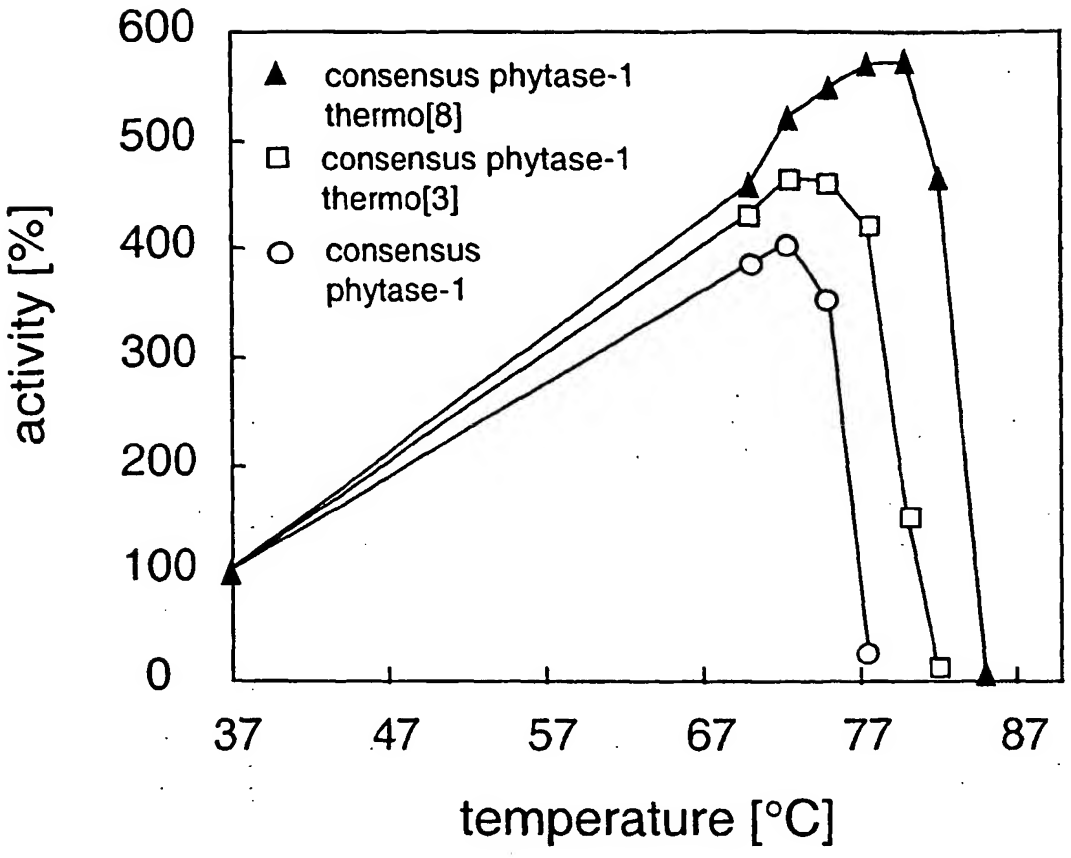

600
500
400
300
200
100
0
activity [%]
consensus phytase-1 thermo[8]
consensus phytase-1 thermo[3]
consensus phytase-1
37
47
57
67
77
87
temperature [°C]

Figure 30

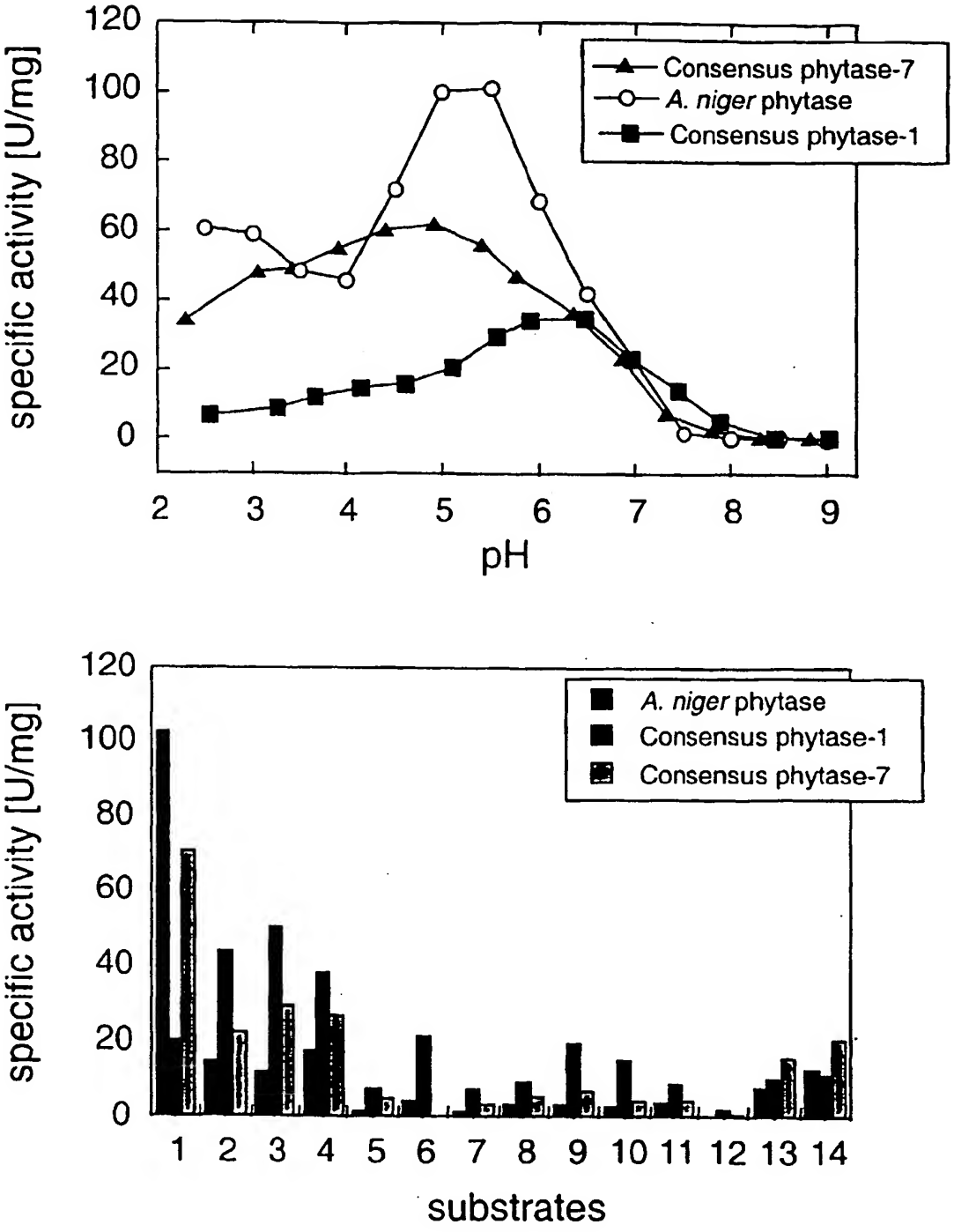

specific activity [U/mg]
120
100
80
60
40
20
0
2
3
4
5
6
7
8
9
pH
Consensus phytase-7
A. niger phytase
Consensus phytase-1
specific activity [U/mg]
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
substrates
A. niger phytase
Consensus phytase-1
Consensus phytase-7

Figure 31

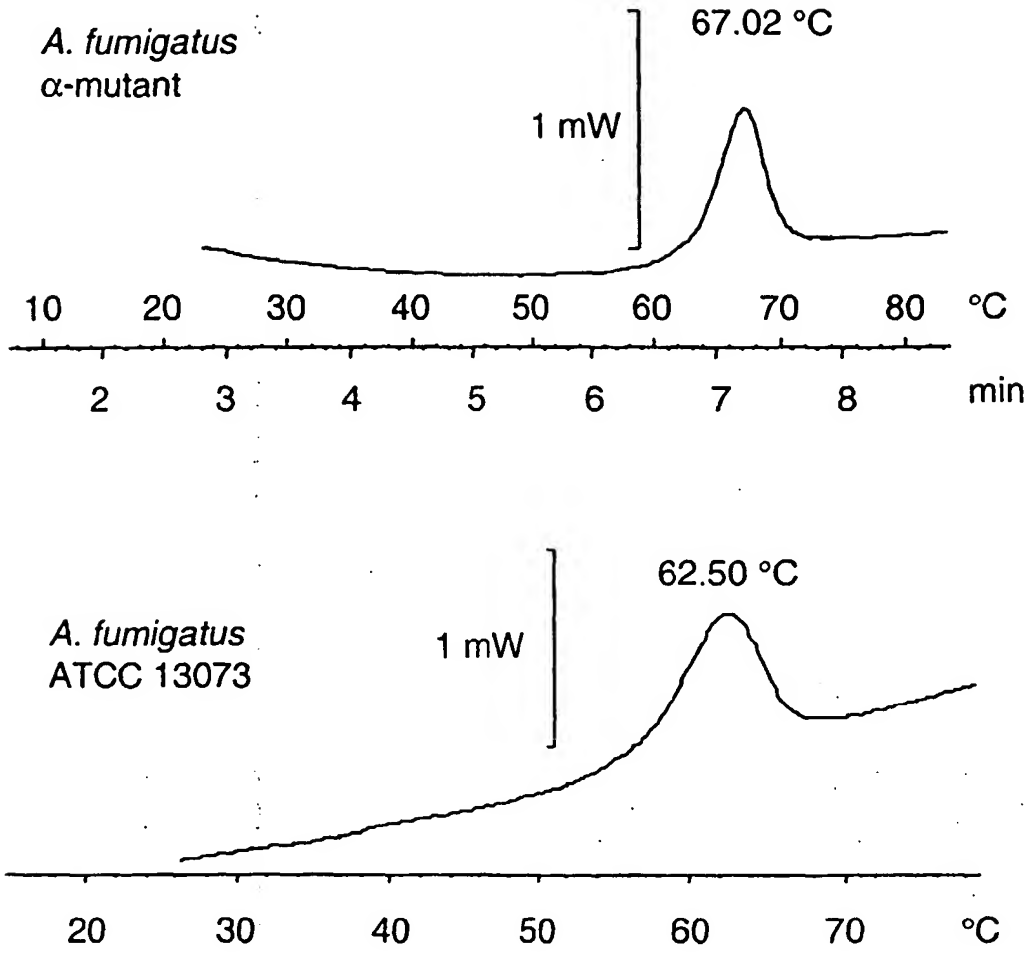

A. fumigatus
α-mutant
67.02 °C
1 mW
10
20
30
40
50
60
70
80
°C
2
3
4
5
6
7
8
min
A. fumigatus
ATCC 13073
62.50 °C
1 mW
20
30
40
50
60
70
°C

Figure 32

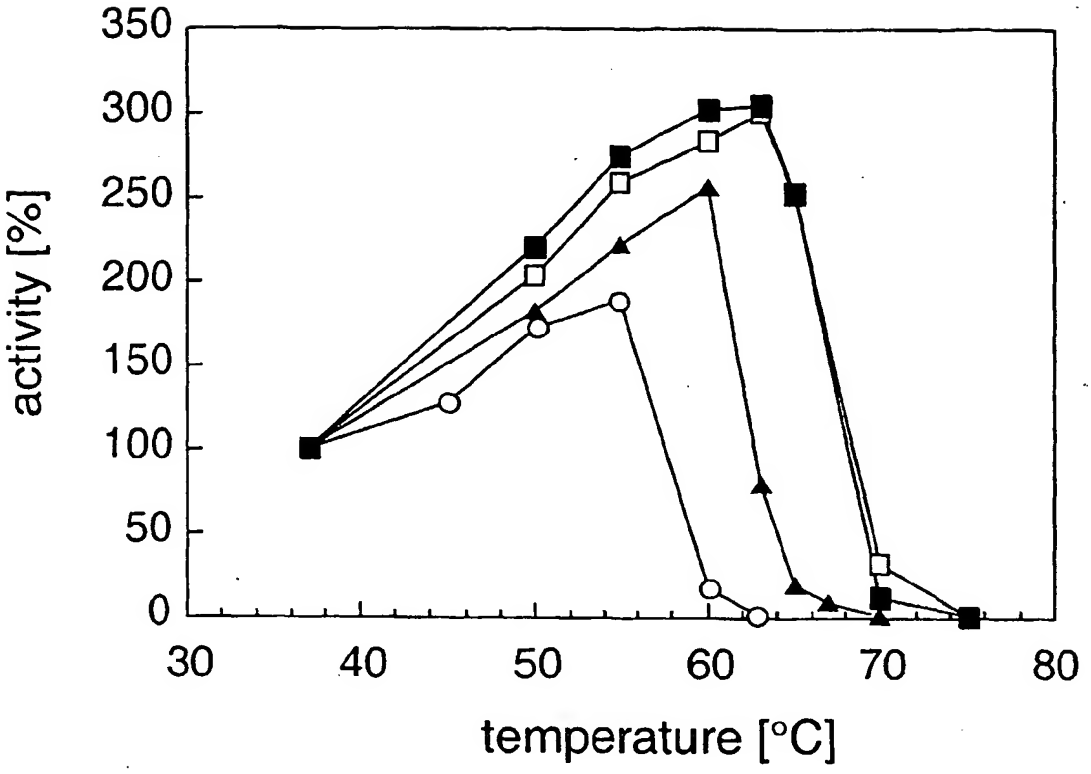

350
300
250
200
150
100
50
0
activity [%]
30
40
50
60
70
80
temperature [°C]

Figure 33

1 MGVFVVLLSI ATLFGSTSGT ALGPRGNSHS CDTVDGGYQC FPEISSNWSP

51 YSPYFSLADE SAISPDVPKG CRVTFVQVLQ RHGARFPTSG AATRISALIE

101 AIQKNATAFK GKYAFLKTYN YTLGADDLVP FGANQSSQAG IKFYRRYKAL

151 ARKIVPFIRA SGSDRVIDSA TNWIEGFQSA KLADPGANPH QASPVINVII

201 PEGAGYNNTL DHGLCTAFEE SELGDDVEAN FTAVFAPPIR ARLEAHLPGV

251 NLTDEDVVNL MDMCPFDTVA RTSDATELSP FCDLFTHDEW IQYDYLGDLD

301 KYYGTGAGNP LGPAQGVGFV NELIARLTHS PVQDHTSTNH TLDSNPATFP

351 LNATLYADFS HDNTMVAIFF ALGLYNGTKP LSTTSVESIE ETDGYSASWL

401 VPFSARMYVE MMQCEAEKEP LVRVLVNDRV VPLHGCGVDK LGRCKRDDFV

451 EGLSFARSGG NWEECFA

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 99 11 1949

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
P,X	WO 98 54980 A (HARZ HANS PETER ;BARENDSE RUDOLF CAROLUS MARIA (NL); GIST BROCADES) 10 December 1998 (1998-12-10) see page 12, lines 14-21; page 13, last paragraph; page 15, last paragraph; ---	1,4,15, 19,20,22	C12N9/96 C12N9/16 A23K1/165
Y	WO 95 00662 A (BOEHRINGER MANNHEIM CORP) 5 January 1995 (1995-01-05) page 2, lines 13-16; ---	1,3-16, 19-22	
Y	EP 0 035 204 A (MILES LAB) 9 September 1981 (1981-09-09) see page 11, line 17 ---	1,3-16, 19-22	
Y	DE 14 92 060 A (NORDMARK-WERKE GMBH) 6 March 1969 (1969-03-06) see page 2, lines 3-6 ---	1,3-16, 19-22	
Y	WO 91 14773 A (CRANFIELD BIOTECHNOLOGY LTD) 3 October 1991 (1991-10-03) see page 2, line 16 ---	1,3-16, 19-22	
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
A	DATABASE WPI Derwent Publications Ltd., London, GB; AN 1997-220413 XP002120048 & JP 09 065877 A (ORIENTAL YEAST CO LTD.), 11 March 1997 (1997-03-11) see the abstract * abstract * ---	2,17,18	C12N A23K
A	KHARE, S.K. ET AL.: "Entrapment of wheat phytase in polyacrylamide gel and its application in soymilk phytate hydrolysis" BIOTECHNOLOGY AND APPLIED BIOCHEMISTRY, vol. 19, - 1994 pages 193-198, XP002120047 see Figures 1 and 2 ---	1-22	
	-/--		

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	25 October 1999	Alt, G

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& : member of the same patent family, corresponding document

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EUROPEAN SEARCH REPORT

Application Number
EP 99 11 1949

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	EP 0 758 018 A (GIST BROCADES BV) 12 February 1997 (1997-02-12) see the whole document	1-22	
P,A	EP 0 897 985 A (HOFFMANN LA ROCHE) 24 February 1999 (1999-02-24) see the whole document	1-22	
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)

The present search report has been drawn up for all claims.

Place of search	Date of completion of the search	Examiner
MUNICH	25 October 1999	Alt, G

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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& : member of the same patent family, corresponding document

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The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

25-10-1999

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9854980 A	10-12-1998	AU 8435798 A	21-12-1998
		AU 8435898 A	21-12-1998
		WO 9855599 A	10-12-1998
WO 9500662 A	05-01-1995	AU 680520 B	31-07-1997
		AU 7116094 A	17-01-1995
		CA 2165663 A	05-01-1995
		EP 0705348 A	10-04-1996
		JP 8511942 T	17-12-1996
		NZ 268301 A	24-10-1997
		US 5627075 A	06-05-1997
EP 0035204 A	09-09-1981	AT 30296 T	15-11-1987
		CA 1187410 A	21-05-1985
		DE 3176491 A	26-11-1987
		DK 98681 A,B,	06-09-1981
		ES 500121 A	01-01-1982
		JP 1980554 C	17-10-1995
		JP 6011702 B	16-02-1994
		JP 56139422 A	30-10-1981
		US 4440679 A	03-04-1984
		US 4623717 A	18-11-1986
DE 1492060 A	06-03-1969	NONE	
WO 9114773 A	03-10-1991	AT 160171 T	15-11-1997
		AU 7572091 A	21-10-1991
		CA 2040815 A,C	20-10-1992
		DE 69128196 D	18-12-1997
		DE 69128196 T	07-05-1998
		EP 0523130 A	20-01-1993
JP 9065877 A	11-03-1997	NONE	
EP 0758018 A	12-02-1997	AU 703007 B	11-03-1999
		AU 6073296 A	06-02-1997
		CA 2182236 A	29-01-1997
		CN 1159208 A	10-09-1997
		CZ 9700874 A	18-03-1998
		WO 9705245 A	13-02-1997
		JP 9154576 A	17-06-1997
		NZ 299071 A	24-09-1998
		PL 319347 A	04-08-1997
		SK 40697 A	08-10-1997
		US 5827709 A	27-10-1998

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The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

25-10-1999

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0897985 A	24-02-1999	CN 1208768 A	24-02-1999
		JP 11146791 A	02-06-1999
		NO 983364 A	25-01-1999

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82